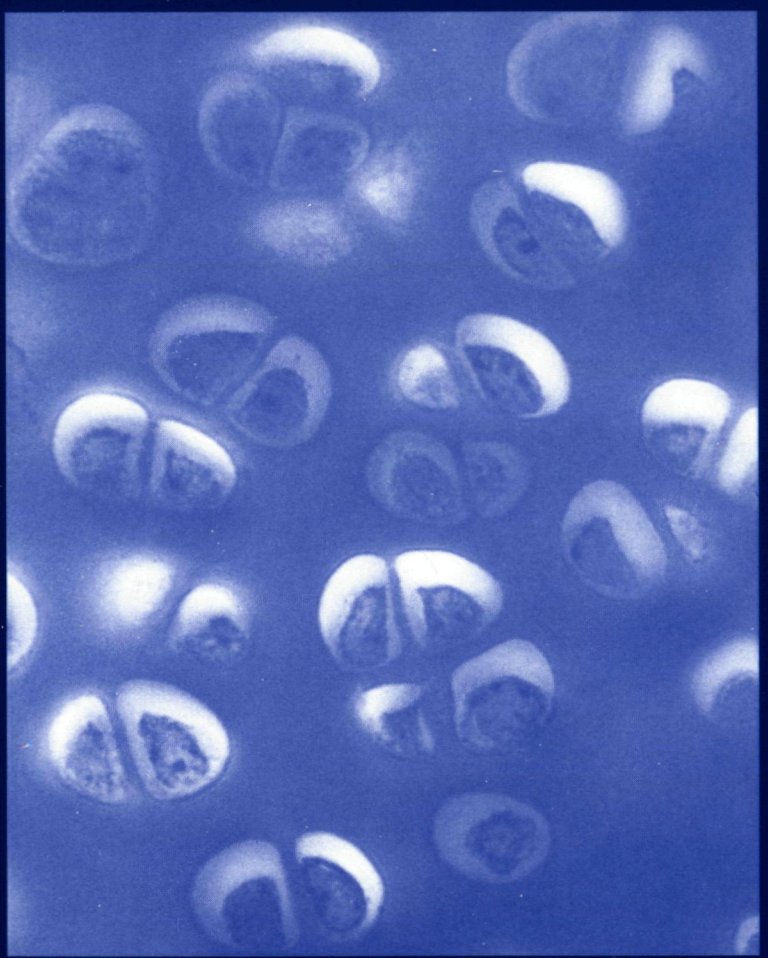
Regulation of articular chondrocyte proteoglycan metabolism by transforming growth factor β and bone morphogenetic protein-2



Harrie Glansbeek

REGULATION OF ARTICULAR CHONDROCYTE PROTEOGLYCAN METABOLISM BY TRANSFORMING GROWTH FACTOR β AND BONE MORPHOGENETIC PROTEIN-2

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CONTENTS

LIST OF ABBREVIATIONS

bFGF	basic fibroblast growth factor
BMP	bone morphogenetic protein
BSA	bovine serum albumin
CPC	cetylpiridinium chloride
DNA	deoxyribonucleic acid
DSS	disuccinimidyl suberate
E. coli	Escherichia coli
EGF	epidermal growth factor
ELISA	enzyme-linked immunosorbent assay
FCS	fetal calf serum
GAG	glycosaminoglycan
IGF-1	insulin-like growth factor-1
IEF	isoelectric focusing
IPTG	isopropyl-β-D-thiogalactopyranoside
IL-1	interleukin-1
kDa	kilodalton
LAP	latency-associated peptide
LTBP	latent TGF-β binding protein
MMP	metalloproteinase
mRNA	message ribonucleic acid
Ni-NTA	nickel-nitrilotriacetic acid
NO	nitric oxide
OA	osteoarthritis
PAI-1	plasminogen activator inhibitor-1
PCR	polymerase chain reaction
PDGF	platelet-derived growth factor
PG	proteoglycan
PMSF	*p*-methyl sulphonyl fluoride
P. pastoris	Pichia pastoris
RA	rheumatoid arthritis
rh	recombinant human
RNA	ribonucleic acid
RT	room temperature
RT-PCR	reverse transcriptase-polymerase chain reaction
SDS-PAGE	sodium dodecyl sulphate-polyacrylamide gel electrophoresis
TIMP	tissue inhibitor of metalloproteinases
TGF-β	transforming growth factor β
TGFβRII	type II TGF-β receptor
TGFβsRII	soluble type II TGF-β receptor
TNFα	tumor necrosis factor α

CHAPTER 1

GENERAL INTRODUCTION,

AIM AND SCOPE OF THE THESIS

GENERAL INTRODUCTION

Articular cartilage

Articular cartilage is a specialized connective tissue that covers the surface of bones in diarthrodial joints. It has an essential function in the resistance of compressive forces and distribution of load. Articular cartilage consists of a dense network of collagen fibrils and a high concentration of aggregating proteoglycans (PG). The collagen fibrils, mainly composed of type II collagen, give the tissue tensile strength while the compressive stiffness of cartilage is the result of the presence of the aggregating proteoglycans (1). Proteoglycans are macromolecules that consist of a core protein with one or more sulfated glycosaminoglycan (GAG) chains. Due to the presence of the highly charged GAG chains, proteoglycans create a high osmotic pressure in the tissue that draws water into the cartilage and causes the tissue to swell (2,3). The large aggregating proteoglycan aggrecan consists of a core protein that can contain up to 150 GAG chains (2,3). The aggrecan monomers are immobilized in the matrix by the formation of large aggregates, created by non-covalent binding of aggrecan monomers (5-800) to hyaluronic acid (2). Besides aggrecan, cartilage contains several small, non-aggregating proteoglycans like decorin, biglycan and fibromodulin (3,4).The different extracellular matrix molecules in articular cartilage are produced by specialized cells, the chondrocytes, which are embedded in the matrix. Chondrocytes are metabolically active cells which continuously synthesize and degrade the extracellular matrix molecules in a coordinated way to maintain healthy cartilage. A schematic illustration of articular cartilage is shown in figure 1.

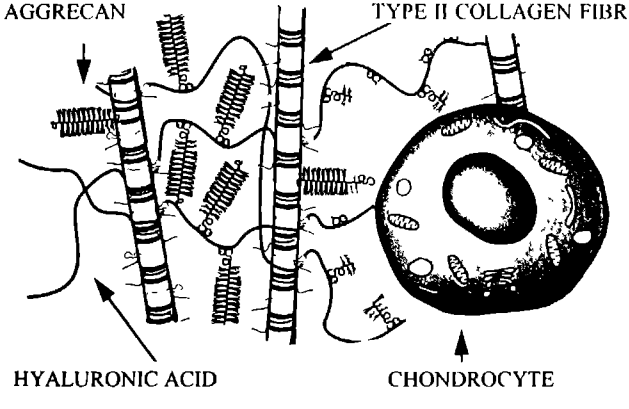

Figure 1 Schematic illustration of articular cartilage. Shown are the chondrocytes, type II collagen fibrils, hyaluronic acid and large aggregates of aggrecan molecules. (Modified illustration from Poole (5))

Cartilage destruction in osteoarthritis and rheumatoid arthritis

Progressive destruction of articular cartilage is a main feature of rheumatoid arthritis (RA) and osteoarthritis (OA). The destruction of articular cartilage will cause functional disability of the joints and ma3y cause invalidity. RA is a joint disease that affects ~1 % of the population and is characterized by chronic inflammation of the joints (6). OA is a joint disease that affects a great part of the population, especially the elderly (7,8). Although synovial hyperplasia and influx of inflammatory cells in the synovial tissue are observed in a significant number of patients (9-11) the disease is, in contrast to RA, primarily non-inflammatory.

Mechanisms of proteoglycan depletion

A main event in the process of articular cartilage destruction in RA and OA is depletion of proteoglycans from the cartilage which is the result of a disbalance between PG synthesis and PG degradation. The enhanced degradation of proteoglycans in RA and OA is mainly caused by action of proteinases but also nonenzymatic agents, such as reactive oxygen metabolites, might be involved (12,13). Until now, it is not clear which enzymes are responsible for the degradation of articular cartilage but there is accumulating evidence that the unidentified enzymatic activity "aggrecanase" plays an important role in the catabolism of aggrecan in OA and RA (14-16). In addition, matrix metalloproteinases (MMPs) seem to be involved in the degradation of cartilage since these enzymes are potent proteinases for extracellular matrix molecules and enhanced activity of these enzymes was shown in joints of patients with RA or OA (14,16-22).

Destruction of articular cartilage in experimental arthritides appears to be not only the result of enhanced degradation of extracellular matrix molecules but also inhibition of PG synthesis contributes to the disbalance between matrix catabolism and anabolism. In contrast to the suppressed PG synthesis observed in arthritis (23,24), PG synthesis in early OA seems to be elevated (11,25,26) but is unable to overcome the enhanced PG degradation.

It is assumed that the cytokines interleukin-1 (IL-1) and tumor necrosis factor α (TNFα) play a key role in the process of PG depletion in articular cartilage since the expression of these cytokines is enhanced in arthritic and osteoarthritic joints (27-31) and these cytokines are able to suppress PG synthesis and to stimulate PG resorption *in vitro* (32-36). The enhanced degradation of proteoglycans by IL-1 and TNFα may be the result of stimulation of MMP synthesis and downregulation of proteinase inhibitors like TIMPs (37-45). In addition, IL-1 can induce aggrecanase activity in articular cartilage (46). Although IL-1 and TNFα can stimulate

PG degradation *in vitro,* their role in PG degradation in humans has to be further elucidated (47-51). However, there is convincing evidence that IL-1 is responsible for the suppression of PG synthesis *in vivo* since several studies have shown that suppression of PG synthesis in experimental arthritides is prevented by neutralization of IL-1 (50-53).

Anabolic factors and cartilage repair

A challenging area of research is the development of a therapy which inhibits cartilage degradation. Since IL-1 and TNFα appear to be key mediators in cartilage destruction, targeting of these factors with antibodies, soluble binding proteins or receptor antagonists might suppress cartilage destruction. Suppression of MMP activity with specific proteinase inhibitors might also be a successful treatment to prevent further cartilage degradation. However, since articular cartilage has a limited capacity for self renewal it is also crucial to develop ways to stimulate cartilage repair. In this respect anabolic factors like insulin-like growth factor-1 (IGF-1)(54-58), platelet-derived growth factor (PDGF)(59-61), basic fibroblast growth factor (bFGF)(62-66), and epidermal growth factor (EGF)(61,67,68) have been studied but the ideal factor to stimulate cartilage repair has not yet been found. Because transforming growth factor-β (TGF-β) and bone morphogenetic protein-2 (BMP-2) seem promising factors to stimulate cartilage repair, the effects of these factors on articular chondrocyte PG metabolism will be addressed in more detail.

Transforming growth factor β

Transforming growth factor β (TGF-β) is a multifunctional growth factor that has profound regulatory effects on many physiological processes like embryonic development, inflammation and tissue repair (69). TGF-β belongs to the transforming growth factor ß (TGF-ß) family. This superfamily consists of a large group of dimeric molecules of which each monomer contains 7 conserved cysteine residues (70). The proteins are synthesized as large precursor proteins. Active TGF-β, a homodimer with a molecular weight of 25 kDa, is generated after activation of the latent complex by enzymatic cleavage (71-73), deglycosylation (71) or exposure to acid environments (74). A schematic representation of TGF-β synthesis and activation is shown in figure 2. Until now, five different isoforms have been identified of which three isoforms (TGF-β1-3) are present in mammals (75). These isoforms have a high degree of homology (75-78) and are conserved between species (75,77-80).

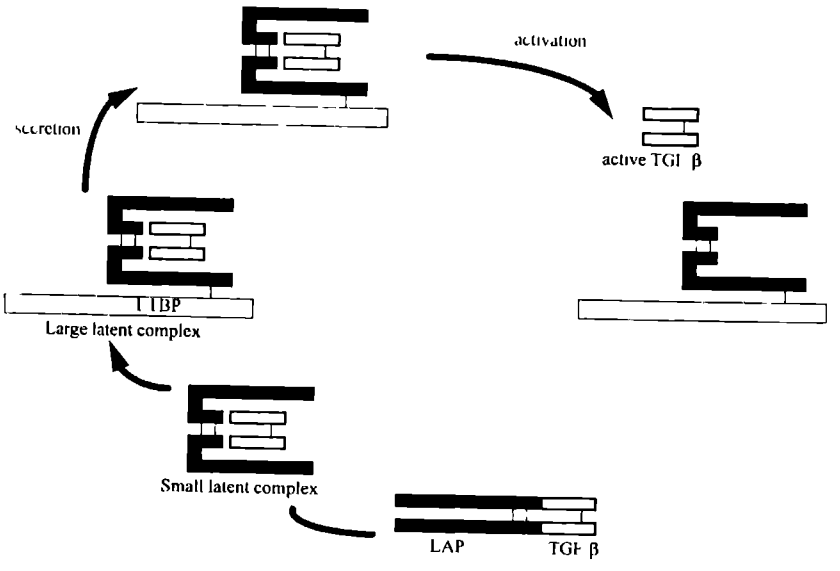

Figure 2 Synthesis and activation of TGF-β TGF-β is synthesized as a large dimeric molecule of which each molecule contains the sequence for latency-associated peptide (LAP) and mature TGF-β After proteolytical cleavage the "Small latent complex" is formed in which LAP and TGF-β are noncovalently associated In most cell types the small latent complex is covalently linked to latent TGF-β binding proteins (LTBP) to form the "large latent complex" This complex is secreted by cells In order to elicit a biological response, the mature protein must be released from the latent complex

TGF-β receptors and signaling

The actions of TGF-β are initiated after binding to specific cell surface receptors Many distinct membrane proteins that can bind TGF-β have been identified (81-84) Articular chondrocytes express at least four classes of TGF-β binding proteins. type I, type II, type III and type V TGF-β receptors (75,85) Type I and type II TGF-β receptors are membrane proteins with an intracellular serine/threonine kinase domain These receptors appear the most important receptors since they are directly involved in signal transduction (84,86). Type III TGF-β receptor, also called betaglycan, is a membrane-bound proteoglycan and is the major TGF-β binding molecule of many cell types (75) It is unlikely that this receptor is directly involved in signal transduction since this protein has a very short intracellular domain (87,88) and cells are described which lack betaglycan but have a normal response on TGF-β (82,89) Although betaglycan is not directly involved in signal transduction it has an important function since it

enhances ligand binding to the signaling receptors (90). This is particularly important for TGF-β2 since cells that express only type I and type II receptors neither bind nor respond efficiently to TGF-β2 (90-92). Type V TGF-β receptor might have a direct function in signal transduction as it contains a serine/threonine kinase domain (93). However, until now evidence for the involvement of type V receptors in signaling is lacking.

As stated before, TGF-β signal transduction is mediated by type I and type II TGF-β receptors. Although initially it was assumed that these receptors mediate distinct signal pathways independently, nowadays there is accumulating evidence that type I and type II TGF-β receptors signal through a heteromeric receptor complex (84,86). The type II receptors bind TGF-β, directly or after presentation by type III receptors, whereafter the complex is recognized by type I receptors and a heteromeric complex is formed (94-96). In this complex the type II receptor, which has constitutive kinase activity (95,97), transphosphorylates the type I receptor (95,98,99) which is the central event in TGF-β receptor activation.

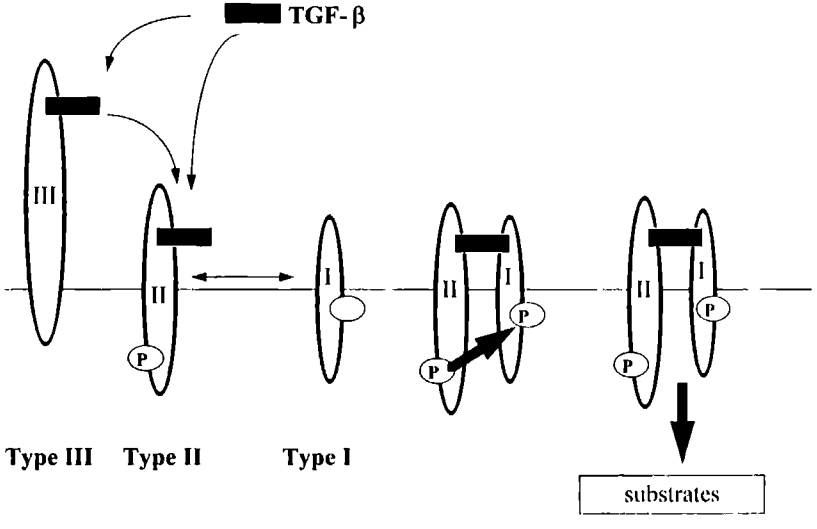

Figure 3 Mechanism of TGF-β receptor binding and signaling. TGF-β binds directly or after presentation by type III TGF-β receptors to the type II TGF-β receptor, whereafter a complex with type I TGF-β receptors is formed. In this complex the constitutive active kinase domain of type II TGF-β receptor phosphorylates the type I TGF-β receptor which is the central event in TGF-β receptor activation. P=active serine/threonine kinase domain.

TGF-β and chondrocyte metabolism

TGF-β is a factor which might have therapeutic value to stimulate cartilage repair. TGF-β is able to stimulate PG synthesis *in vitro* and *in vivo* (100-102) and increases the synthesis of stable PG aggregates through a coordinated increase in hyaluronic acid and PG monomer synthesis (101). The aggrecan molecules produced under TGF-β stimulation are larger and more anionic which might have beneficial effects on tissue function (101,103,104). Besides stimulation of aggrecan synthesis, TGF-β also enhances the expression of biglycan (104,105). Moreover, TGF-β might enhance the retention of newly synthesized matrix molecules by increasing the expression of integrins on chondrocytes (106-108).

The effects of TGF-β on chondrocyte PG synthesis seem to be different between normal articular cartilage and PG depleted cartilage. For example, TGF-β stimulated PG synthesis of human OA cartilage while under the same conditions TGF-β did not affect the PG synthesis of normal articular cartilage (109). In addition, TGF-β enhanced the PG synthesis of IL-1 treated cartilage while the effects of TGF-β on normal cartilage were minimal (110,111). These differential responses between normal and PG depleted cartilage might be related to differences in chondrocyte phenotype and/or differences in TGF-β receptor expression.

An interesting feature of TGF-β is the ability to counteract IL-1-induced suppression of articular cartilage PG synthesis (110,112). The mechanism of this action is unclear but the abolishment of IL-1-effects might be the result of TGF-β-induced down-regulation of IL-1 receptors (113,114) or suppression of nitric oxide (NO) synthesis (115,116), a factor involved in IL-1-induced suppression of PG synthesis (117,118). Since IL-1 is responsible for the suppression of articular chondrocyte PG synthesis in experimental arthritis (50-53), counteraction of IL-1-induced suppression of articular cartilage PG synthesis appears to be an important feature to stimulate cartilage repair in arthritic joints.

Besides the effects on articular cartilage PG synthesis, TGF-β is also a potent regulator of matrix degradation. TGF-β has been shown to inhibit matrix degradation (110,119) probably by down regulating the synthesis of matrix degrading enzymes (120-122) and up-regulation of tissue inhibitor of metalloproteinases (TIMP) (120,122-124). TGF-ß-mediated up-regulation of plasminogen activator inhibitor-1 (PAI-1) (125,126) might also play a role since plasmin has been suggested to be involved in cartilage degradation by direct cleavage of matrix molecules or by the activation of latent metalloproteinases (127-129). The property to stimulate articular cartilage PG synthesis, to counteract IL-1-induced suppression of PG synthesis and to suppress matrix degradation makes TGF-β a promising factor to stimulate articular cartilage repair.

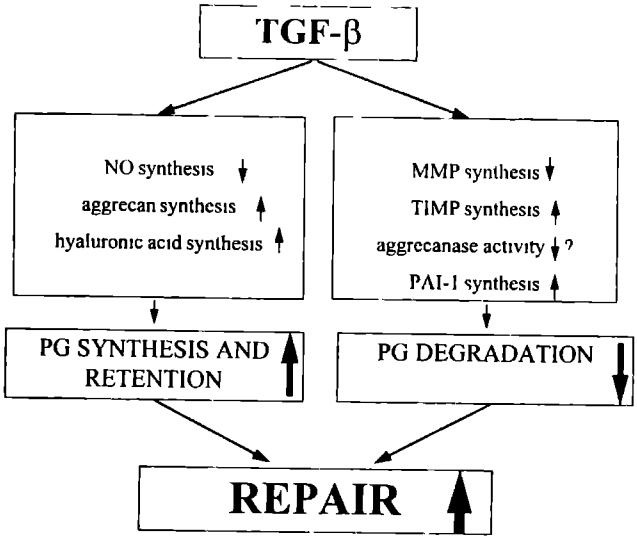

Figure 4 Schematic representation of mechanisms involved in TGF-β-mediated stimulation of cartilage repair.

Bone morphogenetic protein-2 (BMP-2)

BMP-2 belongs to the family of the bone morphogenetic proteins (BMPs). This family belongs, like TGF-β to the TGF-β superfamily. BMPs can be distinguished from other members of the TGF-β superfamily by having 7, rather than 9, conserved cysteines in the mature protein (130). At this moment ten different BMPs have been described of which nine belong to the TGF-β superfamily (130,131). In contrast to the other BMPs, BMP-1 does not belong to the TGF-β superfamily but is a member of the astacin family of metalloproteinases (132,133). BMPs signal, like TGF-β, through heteromeric complexes of type I and type II serine/threonine kinases. Specific type I and type II BMP receptors have been described (134-138). BMPs were first identified on the basis of their ability to induce ectopic bone but nowadays it is known that BMPs are involved in many physiological processes. BMPs play a prominent role in embryogenesis since they are involved in skeletal development, neural patterning and development of organs including kidney, lung, heart and skin (130).

BMP-2 and chondrocyte metabolism

BMP-2 might be an interesting factor to stimulate cartilage repair since this factor has been shown to be a very potent stimulator of articular cartilage PG synthesis *in vitro* (139,140). In addition, BMPs have the potential to stimulate PG synthesis in the presence of IL-1 since IL-1-induced suppression of PG synthesis was counteracted *in vitro* by BMP-7 (141). Members of the BMP family are also able of inhibiting PG degradation. BMP-7 suppressed IL-1-induced up-regulation of collagenase (MMP-1) mRNA and stromelysin (MMP-3) mRNA and counteracted the IL-1-induced inhibition of their natural inhibitor (TIMP)(142). In addition, both BMP-3 and BMP-4 have been shown to inhibit PG degradation in cartilage explants cultured *in vitro* (143). Although it has been reported that BMP-2 is a potent regulator of cartilage PG synthesis *in vitro,* no *in vivo* data are available. In addition, nothing has been reported about the ability of BMP-2 to counteract IL-1 effects on chondrocyte metabolism or about the effects of BMP-2 on articular cartilage PG degradation.

AIM AND SCOPE OF THE THIS THESIS

The aim of this thesis was to get more insight in the regulatory role of transforming growth factor-β (TGF-β) and bone morphogenetic protein-2 (BMP-2) on articular cartilage PG metabolism and to study whether these factors are able to stimulate articular cartilage repair *in vivo*.

The reported effects of TGF-β on articular chondrocytes are contradictory since both stimulating and inhibitory effects of TGF-β on chondrocyte PG synthesis have been described (101,144,145). Therefore, studies were performed to investigate whether differential effects of TGF-β on chondrocytes are related to differences in the expression of TGF-β receptors. As described in **Chapter 2** and **Chapter 3** differential responses of chondrocytes on TGF-β appear not to be correlated with differences in the relative expression of two isoforms of the type II TGF-β receptor ($TGF\beta RII_1$ and $TGF\beta RII_2$) but a relation was found between the response on TGF-β and the size of the type II TGF-β receptors.

Both BMP-2 and TGF-β had been shown to stimulate articular cartilage PG synthesis *in vitro* (100-102,139,140). To study the effects of these factors on PG synthesis *in vivo*, intra-articular injections were given in murine knee joints whereafter PG synthesis was analyzed. As described in **Chapter 4,** both TGF-β and BMP-2 appeared to be very potent stimulators of articular chondrocyte PG synthesis *in vivo*. However, up-regulation of PG synthesis by TGF-β lasted much longer than up-regulation by BMP-2.

IL-1 appears to be a key mediator of cartilage destruction (25,32,50,51,146). Therefore, the ability of BMP-2 and TGF-β to counteract IL-1-effects on cartilage PG synthesis and content was studied. As described in **Chapter 5**, TGF-β counteracted the effects of IL-1 on PG synthesis and content. In contrast to TGF-β, BMP-2 did not show any effect on chondrocyte PG metabolism in the presence of IL-1.

Subsequently, the ability of TGF-β and BMP-2 to stimulate articular cartilage repair in established arthritis was studied (**Chapter 6**). Local administration of BMP-2 in arthritic joints did not show any effect on cartilage PG synthesis or content. In contrast, intra-articular injection of TGF-β clearly stimulated articular cartilage repair. TGF-β did not affect inflammation but stimulated chondrogenesis in the joints.

To elucidate the significance of possible side-effects, long-term effects of local administration of TGF-β into normal murine knee joints were studied (**Chapter 7**). One month after three intra-articular injections of TGF-β pathological changes, similar to changes found in early stages of murine OA (102,147), were found. These data indicate that care must be taken with application of TGF-β and that TGF-β might play a role in the induction of OA.

To further investigate the role of endogenous TGF-β in arthritis and osteoarthritis potent inhibitors for TGF-β are required. The type II TGF-β receptor has a high affinity for TGF-β (94,95,148). Therefore a soluble type II receptor was expressed in *Escherichia coli* and *Pichia pastoris*. In **Chapter 8** the expression, purification and characterization of this protein is described.

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CHAPTER 2

CORRELATION OF THE SIZE OF TYPE II TRANSFORMING GROWTH FACTOR-β (TGF-β) RECEPTOR WITH TGF-β RESPONSES OF ISOLATED BOVINE ARTICULAR CHONDROCYTES

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ABSTRACT

Objective Transforming growth factor ß (TGF-ß) is a multipotent regulator of cell proliferation and extracellular matrix production. The effect of TGF-ß on chondrocyte matrix production was studied in relation to expression of TGF-ß binding proteins. The effect of TGF-ß on proteoglycan (PG) synthesis of isolated articular chondrocytes depended on the culture period. PG synthesis of chondrocytes which were cultured for one day was inhibited by TGF-ß whereas PG synthesis of chondrocytes, cultured in monolayer for seven days or longer, was stimulated by TGF-ß. To investigate if this differential response is related to a distinct expression of TGF-ß receptors, this parameter was studied by affinity labeling.

Methods Chondrocytes were incubated with 100 pM TGF-ß labeled with iodine-125. Crosslinking was performed using 0.25 mM disuccinimidyl suberate (DSS). Membrane proteins were extracted and analysed by denaturating sodium dodecylsulphate polyacrylamide gel electrophoresis (SDS-PAGE) and autoradiography.

Results Freshly isolated and cultured chondrocytes expressed types I, II and III TGF-ß receptors. The type II TGF-ß receptor of cultured chondrocytes appeared to be about 15 KDa smaller than the type II TGF-ß receptor expressed on freshly isolated chondrocytes, however.

Conclusions As the type II TGF-ß receptor appears to be involved in signal transduction, this change in size of the type II TGF-ß receptor might be related to the differential effect of TGF-ß on PG synthesis of freshly isolated and cultured bovine articular chondrocytes.

INTRODUCTION

Transforming growth factor ß (TGF-ß) is a multipotent regulator of cell proliferation and extracellular matrix synthesis (1,2). It is produced as a latent, high molecular weight complex (2,3) which can be activated by proteolytic cleavage or extremes of pH (2). Active TGF-ß is a 25 kDa polypeptide, consisting of two identical subunits of 112 amino-acids, linked by disulfide bonds (1,2). TGF-ß might be an important regulator of articular chondrocyte metabolism during joint diseases, as high concentrations of latent TGF-ß (300 ng/g) are present in articular cartilage (4) and active TGF-ß is found in the synovial fluid of patients with rheumatoid arthritis or osteoarthritis (5).

The reported effects of TGF-ß on chondrocyte metabolism are contradictory. Stimulating and inhibitory effects of TGF-ß on proteoglycan (PG) and DNA synthesis of articular chondrocytes have been described (6-9). We have shown that effects of TGF-ß on PG and DNA synthesis of isolated bovine articular chondrocytes are related to differences in culture time (10). TGF-ß inhibits PG and DNA synthesis of isolated bovine articular chondrocytes which were cultured for one day ('freshly isolated chondrocytes'), whereas TGF-ß stimulates PG and DNA synthesis of bovine articular chondrocytes cultured for seven days or longer ('cultured chondrocytes'). This effect appeared to be the result of phenotypical changes between freshly isolated and cultured chondrocytes.

Effects of TGF-ß are mediated by cell surface receptors. Articular chondrocytes express at least four distinct types of TGF-ß binding proteins: type I (53 KDa), type II (70-100 KDa), type III (250-350 KDa) and type V (400 KDa)(1,11). Other types of TGF-ß binding proteins, like the type VI TGF-ß receptor, are described on several cell types (12-14), but it is not known if these are also present on chondrocytes. Little is known about the function of these different binding proteins, though on a large variety of cell types it was demonstrated that the type I and II receptors are important in signal transduction (15-21). Involvement of the type II TGF-ß receptor in signal transduction is also suggested by the presence of a serine/threonine kinase domain (22). The type V receptor might also have a function in signal transduction since it contains a serine/threonine kinase domain (23) and TGF-ß resistant tumor cell lines have been described which have no expression of type V receptors but a normal expression of the other TGF-ß receptors (11). The type III receptor, also called betaglycan, probably does not have a role in signal transduction as it has a small intracellular domain (24,25) and

cells are described which lack betaglycan but have a normal response on TGF-ß (26,27).

The aim of this study was to investigate if the differential effect of TGF-ß on PG synthesis of freshly isolated and cultured articular chondrocytes is related to differences in TGF-ß receptor expression.

MATERIALS AND METHODS

Isolation of bovine articular chondrocytes

Articular cartilage chondrocytes were isolated from bovine metacarpophalangeal joints. Cartilage slices were incubated for 48 hours in RPMI DM (Flow Laboratories) supplemented with 1 mg/ml Clostridium collagenase (371 Units/mg, Worthington Biochemical Corp.) at 37 °C in a humidified 5% CO_2 atmosphere. After incubation with collagenase, chondrocytes were washed three times with collagenase free medium. Cells were seeded at a density of 5 x 10^5 cells/ml in 24 well cluster dishes (1ml/cluster, Costar) and grown for 1 day ('freshly isolated chondrocytes') or 14 days ('cultured chondrocytes') in medium supplemented with 20% fetal calf serum (FCS)(Flow Laboratories). Culture medium was changed every other day. The cell number doubled during culture in approximately one week after which confluence was reached.

Effect of TGF-ß on proteoglycan synthesis

Recombinant TGF-ß1 (Serva) was solubilized in 4 mM HCl with 0.1% bovine serum albumin (Sigma). Freshly isolated and cultured chondrocytes were incubated for 24 hours in the presence of TGF-ß (5 ng/ml, 200 pM). Four hours before the end of the incubation period, 10 μCi ^{35}S-sulfate (Du Pont de Nemours) was added. After the incubation period, the culture medium was treated with papain (1mg/ml papain, (Sigma), 0.2 M NaCl, 0.1 M Na-acetate, 10 mM L-cysteine hydrochloride (Sigma), 50 mM Na_2EDTA, 50 μg/ml chondroitin sulfate carrier (Sigma), pH 6.0) for 2 hours at 60 °C. The glycosaminoglycans (GAG) were precipitated by incubation (2 hours, 37 °C) with 0.1% cetylpiridinium chloride (CPC, Sigma). After centrifugation (15 minutes, room temperature, 10.000 g) the pellet was washed three times with 0.05% CPC. The pellet was solubilized for 2 h at 60°C with lumasolve (Perstorp Analytical) and after addition of scintillation fluid counted in a liquid scintillation counter.

The Wilcoxon's Rank Sum test was used to test statistical significance. Differences were significant when the P value was less than 0.05.

Collagenase treatment of cultured chondrocytes

Chondrocytes, cultured for 14 days in medium supplemented with 20% fetal calf serum (FCS), were incubated for 48 hours in RPMI DM supplemented with 1 mg/ml Clostridium collagenase (371 Units/mg, Worthington Biochemical Corp.) at 37 °C in a humidified 5% CO_2 atmosphere. After incubation with collagenase, chondrocytes were washed three times with collagenase free medium. Cells were seeded at a density of 5 x 10^5 cells/ml in 24 well cluster dishes (1ml/cluster) and grown for one day, after which the effect of TGF-ß on the PG synthesis was measured.

^{125}I Labeling of TGF-β

TGF-ß1 was labeled with iodine-125 using the oxidizing agent 1,3,4,6-tetrachloro-3α,6α-diphenyl glycoluril (Iodogen; Pierce) according to the method described by Salacinski *et al* (28). 5 μg TGF-ß in 100 μl sodium phosphate buffer (0.05M, pH 7.4) was incubated in the presence of 0.5 mCi carrier free $Na^{125}I$ (Amersham) in a reaction vial coated with 2 μg Iodogen. After an incubation of 15 minutes at room temperature, the reaction was terminated by addition of 1 M KI. Radiolabeled TGF-ß was separated from free iodine using a Sephadex G25 column which was equilibrated with 4 mM HCL containing BSA (0.1% w/v).

Affinity labeling

Affinity labeling was performed according to the method of Massagué and Like (29). Chondrocytes were washed with binding buffer (128 mM NaCl, 5 mM KCl, 1.2 mM $CaCl_2$, 1.2 mM MgSO , 50 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonate, pH 7.5) after which they were incubated with 100 pM ^{125}I-TGF-ß in binding buffer, supplemented with 5 mg/ml BSA, for 3 hours at 4 °C. Competition studies were performed in the presence of 4 nM non-radiolabeled TGF-ß. After the incubation period the buffer was discarded and the crosslink reaction was performed in binding medium supplemented with 0.25 mM disuccinimidyl suberate (Pierce) for 30 minutes at 4°C. After washing cells with binding buffer the cells were incubated (16 hours, 4 °C) with extraction buffer (1% v/v Triton X-100, 1 mM EDTA, 10 mM Tris, and 1 mM *p*-methyl sulphonyl fluoride (PMSF). Detergent soluble material was analysed by denaturating SDS-polyacrylamide gel electrophoresis, using prefababricated 5-20% gradient gels (Bio-Rad), and autoradiography. Autoradiograms were scanned using an automatic gel

scanner (LKB Ultroscan XL). Sizes of binding proteins were determined using pre-stained molecular weight markers (Bio-Rad).

RESULTS

As shown previously (10), TGF-ß inhibited PG synthesis of freshly isolated chondrocytes whereas PG synthesis of cultured chondrocytes was stimulated by TGF-ß (Figure 1). It was suggested that this phenomenon is the result of phenotypical changes occurring during culture. However, the possibility that the lack of stimulation on freshly isolated chondrocytes was a result of the collagenase treatment, which was used to isolate chondrocytes from cartilage, still remained. To exclude this possibility, cultured chondrocytes were treated with collagenase, seeded in the original density of 5 x 10^5 cell/ml and cultured for 24 hours after which the effect of TGF-ß on the PG synthesis was measured. As shown in figure 1, collagenase treatment of cultured chondrocytes did not alter the effect of TGF-ß, showing that the differential effect of TGF-ß was indeed a result of phenotypical changes.

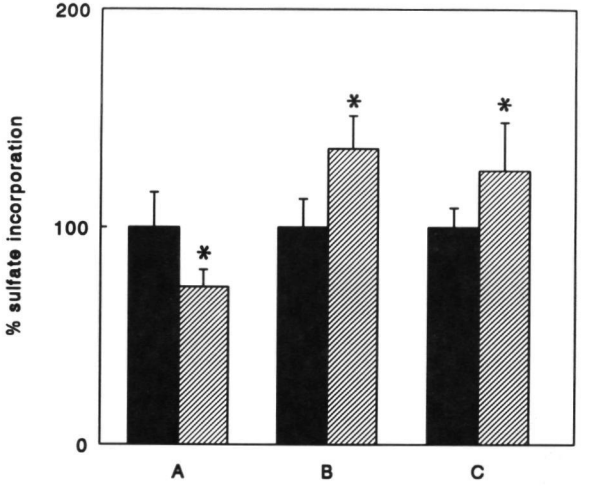

Figure 1 *Effect of TGF-ß on the PG synthesis of freshly isolated articular chondrocytes (A), cultured chondrocytes (B) and cultured chondrocytes after collagenase treatment (C). Chondrocytes were incubated with (hatched bar) or without (solid bar) 200 pM TGF-ß for 24 hours. During the last 4 hours the culture medium was supplemented with 10 µCi ^{35}S-sulfate. After incubation PGs were precipitated and ^{35}S content was measured. Experiments were performed in quintuple. Without a TGF-ß incubation the ^{35}S incorporation of cultured chondrocytes and cultured chondrocytes after collagenase treatment, were comparable to the ^{35}S incorporation of freshly isolated chondrocytes. Shown are the means (+SD) of a representative experiment of four. Significant differences are indicated with asterisk.*

To investigate if this effect was related to alterations in TGF-ß receptor expression, affinity labeling was performed. After SDS-PAGE and autoradiography, several bands were demonstrated. The sizes of these bands correspond with sizes of the affinity-labeled type I (65 kDa), type II (85 kDa) and type III (diffuse high molecular band 250-350 kDa) TGF-ß receptors (Figure 2). It might be possible that the diffuse high molecular band also includes the affinity-labeled types V (400 kDa) and VI (180 kDa) binding proteins, but that the separation capacity in this region of the gel was not enough to distinguish them from the type III receptor. Studies in which affinity labeling was performed in the presence of 4 nM non-radiolabeled TGF-ß showed that these affinity-labeled proteins are specific TGF-ß binding proteins as competition between ^{125}I-TGF-ß and non-radiolabeled TGF-ß for binding to these receptors was demonstrated (Figure 2). In addition to the types I, II and III TGF-ß receptors, we showed a band of about 30 kDa, corresponding to a binding protein of 18 kDa. This binding protein appears not to be a specific TGF-ß binding protein, as we could not show competition between radiolabeled and non-radiolabeled TGF-ß for binding to this protein (Figure 2).

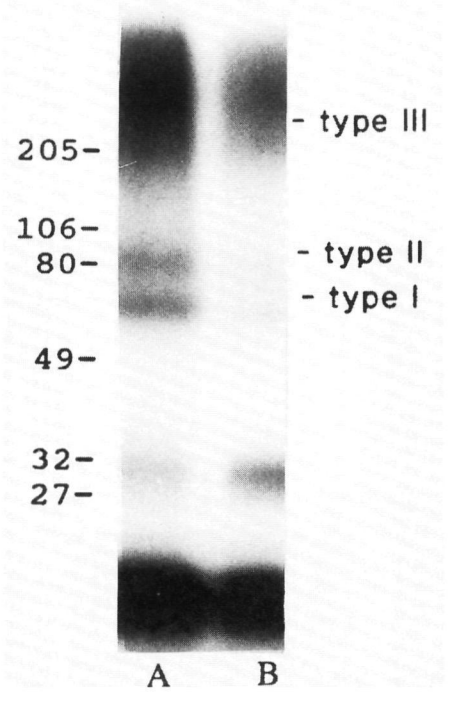

Figure 2 *Affinity labeling of cultured chondrocytes without* ***(A)*** *and in the presence* ***(B)*** *of 4 nM non-radiolabeled TGF-β. Chondrocytes were incubated with 100 pM ^{125}I-TGF-β. Crosslinking was performed using 0.25 mM disuccinimidyl suberate. Membrane proteins were extracted and analyzed by denaturating SDS-PAGE and autoradiography. Molecular weight markers and bands corresponding with the affinity-labeled type I, type II and type III TGF-β receptors are indicated.*

Although isolated and cultured chondrocytes expressed the 18 kDa binding protein and types I, II and III TGF-ß receptors, a difference in TGF-ß receptor expression was demonstrated between these chondrocytes. A small, but significant difference was observed in the size of the type II TGF-ß receptors, whereas no other reproducible differences were observed in TGF-ß receptor expression. The type II TGF-ß receptor of freshly isolated chondrocytes appears to be about 15 kDa larger than the type II receptor of cultured chondrocytes (Figure 3). The variation in size of the type II receptor was also demonstrated after scanning autoradiograms with an automatic gel scanner (Figure 4). The ratio between the intensities of bands showed variation between experiments. We could not show a correlation between the ratio of the intensities of the type I and type II receptors and the effects of TGF-ß on chondrocytes, however. The TGF-ß receptor expression of cultured chondrocytes which were treated with collagenase was also analysed. We were not able to show differences between TGF-ß receptor expression of cultured chondrocytes and cultured chondrocytes which were treated with collagenase (data not shown).

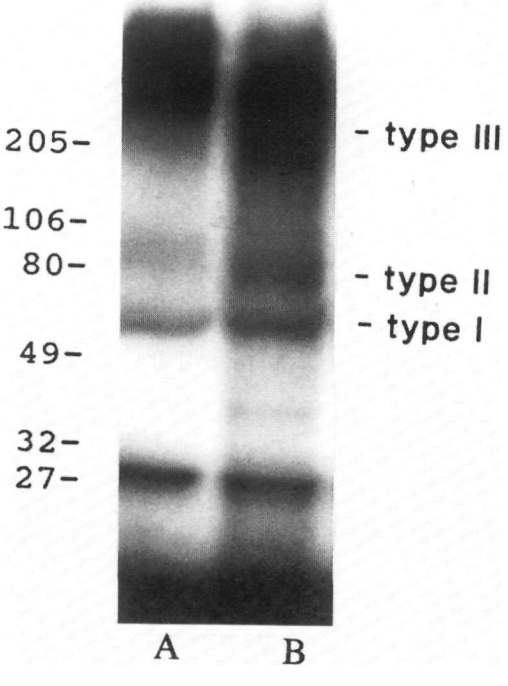

Figure 3 *Affinity labeling of freshly isolated* ***(A)*** *and cultured* ***(B)*** *chondrocytes. Chondrocytes were incubated with 100 pM ^{125}I-TGF-ß. Crosslinking was performed using 0.25 mM disuccinimidyl suberate. Membrane proteins were extracted and analyzed by denaturating SDS-PAGE and autoradiography. Molecular weight markers and bands corresponding with the affinity labeled type I, type II and type III TGF-ß receptors are indicated. Shown is a representative autoradiogram of three.*

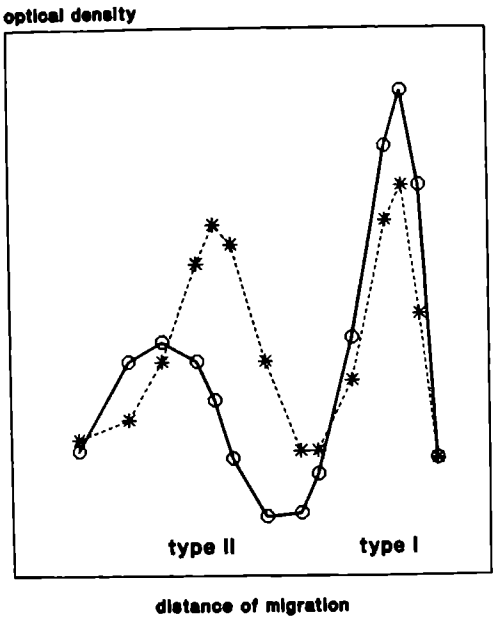

*Figure 4 Scan of autoradiogram. Autoradiograms were scanned using an automatic gelscanner. Absorbtion units were normalized for background. The relative positions of the type I and type II TGF-β receptors of freshly isolated (O) and cultured (*) chondrocytes are shown.*

DISCUSSION

Proteoglycan synthesis of isolated articular chondrocytes has been reported to be both stimulated and inhibited by TGF-ß (6-9). We have shown before that these differential findings may be the result of differences in culture period (10). PG synthesis of bovine articular chondrocytes which were cultured for one day ('freshly isolated chondrocytes'), was inhibited, whereas the PG synthesis of chondrocytes cultured for seven or more days ('cultured chondrocytes') was stimulated by TGF-ß. In this study we excluded the possibility that this was an effect of the collagenase treatment used to isolate chondrocytes from cartilage, suggesting that the differential effect of TGF-ß on freshly isolated and cultured chondrocytes is a result of phenotypical changes occurring during culture. We showed before that freshly isolated chondrocytes are normal differentiated chondrocytes since they exhibited the typical polygonal morphology of differentiated chondrocytes and produced predominantly large proteoglycans, both characteristics of differentiated chondrocytes (10). During culture, phenotypical changes occur as monolayer cultured chondrocytes had an altered morphology and

synthesized besides large proteoglycans, a considerable quantity of smaller proteoglycans.

The observation that PG synthesis of normal differentiated chondrocytes is inhibited by TGF-ß is in agreement with earlier observations (6,30). PG synthesis of articular chondrocytes cultured in agarose, a system in which chondrocytes maintain their differentiated phenotype, was inhibited by TGF-ß (6). Moreover, PG synthesis of intact murine articular cartilage, cultured for one day, was also inhibited by TGF-ß (30). Since effects of TGF-ß are mediated by its cell surface receptors, we investigated whether the dissimilar effect of TGF-ß on PG synthesis of freshly isolated and cultured chondrocytes could be the result of differences in TGF-ß receptor expression.

Using affinity labeling we were able to show that the type II TGF-ß receptor of cultured chondrocytes is about 15 kDa smaller than the type II TGF-ß receptor of freshly isolated chondrocytes. No other reproducible variations in TGF-ß receptor expression were found. This observation suggests a relation between the size of the TGF-ß type II receptor and the effect of TGF-ß on the PG synthesis of articular chondrocytes. As the type II TGF-ß receptor has been shown to play a part in mediating inhibition of PG synthesis on chondrocytes (31,32), we postulate that the small-sized type II TGF-ß receptor on cultured chondrocytes is non-functional, leading to stimulating effects of TGF-ß by way of the type I or other TGF-ß receptors. The existence of comparable non-functional, small-sized type II TGF-ß receptors on other cell types has been reported earlier. Resistance to growth inhibition by TGF-ß was correlated with the expression of non-functional small-sized type II TGF-ß receptors on mutant bovine endothelial cells (33), mutant mink lung epithelial cells (19) and human colon carcinoma cells (34). As the TGF-ß type II receptor is a glycoprotein, with about 15 kDa of N-linked carbohydrate (1,35), it is possible that the difference in molecular weight between normal and small-sized type II receptors is a result of alterations in glycosylation. Normal glycosylation was demonstrated on the altered type II TGF-ß receptors on mink lung epithelial cells (35), however, suggesting that other mechanisms may be involved. The difference between normal and small-sized type II receptors might also be the result of alternative splicing. Alternative splicing is described for the type II activin receptor (36), a receptor like the type II TGF-ß receptor, belonging to the serine/threonine kinase family (13).

In this study we demonstrated a small-sized type II TGF-ß receptor on phenotypically altered chondrocytes. Stimulation of the PG synthesis of these chondrocytes appears to be correlated with the expression of this small-sized receptor. Because phenotypically changed chondrocytes are also present in osteoarthritic (OA) cartilage (37-39) and the PG synthesis of human OA cartilage is stimulated by TGF-ß, whereas under the same conditions TGF-ß had no effect on the PG synthesis of normal human cartilage (40), non-functional, small-sized type II TGF-ß receptors might be expected in OA cartilage. The increased PG synthesis, characteristic of OA cartilage (41,42), might be the result of the expression of non-functional, small-sized type II TGF-ß receptors in combination with functional type I TGF-ß receptors. Alteration of expression and function of TGF-ß receptors could be a mechanism of chondrocytes in pathological cartilage to initiate the repair process. Interference with cellular physiology by pharmacological modulation of TGF-ß receptor expression might provide new directions for stimulation of tissue repair.

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CHAPTER 3

SPECIES SPECIFIC EXPRESSION OF TYPE II TGF-ß RECEPTOR ISOFORMS BY ARTICULAR CHONDROCYTES: EFFECT OF PROTEOGLYCAN DEPLETION AND AGING.

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ABSTRACT

Recently a new isoform of the type II TGF-ß receptor (TGFßRII) was identified. This isoform ($TGF\beta RII_2$) contains an insertion of 25 aminoacids in the extracellular domain of the receptor. Using RT-PCR we demonstrated that both $TGF\beta RII_1$ and $TGF\beta RII_2$ are expressed by chondrocytes in murine and human articular cartilage. Bovine articular chondrocytes did express $TGF\beta RII_1$ mRNA but did not express detectable levels of $TGF\beta RII_2$ mRNA suggesting that the new isoform does not play an important role in normal bovine cartilage physiology. Because TGF-ß responses seem to be age-related and differential TGF-ß responses have been described between normal cartilage and cartilage undergoing repair we studied if the relative mRNA expression between these isoforms is altered during cartilage repair and aging. No differences in the relative mRNA expression of the two isoforms of the type II TGF-ß receptor could be demonstrated in murine cartilage during aging or during the repair phase after mild PG depletion indicating that it is unlikely that age-related TGF-ß responses and differential TGF-ß responses between normal cartilage and cartilage undergoing repair are the result of differences in the relative expression of the two TGFßRII isoforms.

INTRODUCTION

Transforming growth factor beta (TGF-ß) is a potent regulator of cell metabolism (1,2). A role of TGF-ß in cartilage physiology is suggested by the presence of high concentrations of TGF-ß in normal articular cartilage (3) and its regulatory role in chondrocyte proliferation, differentiation and extracellular matrix production (4-11). Since TGF-ß expression in the joint is enhanced in patients with rheumatoid arthritis (12,13) and active TGF-ß is present in the synovial fluid of patients with rheumatoid arthritis or osteoarthritis (14) it is assumed that TGF-ß also plays a role in the regulation of chondrocyte metabolism during these joint diseases.

TGF-ß responses are mediated by specific cell surface receptors. Articular chondrocytes express at least four distinct types of TGF-ß receptors: type I, type II, type III and type V TGF-ß receptors (1,15). The type I and type II TGF-ß receptors seem to be the most important TGF-ß receptors since they are directly involved in signal transduction (16-20).

Recently a new isoform of the type II TGF-ß receptor was isolated from a mouse brain cDNA bank (21), a human glioblastoma cDNA bank (22) and from a human endothelial cell cDNA bank (23). This new isoform ($TGF\beta RII_2$) contains an insertion of 25 amino acids in the extracellular domain of the receptor. It is most likely that this isoform is generated by differential splicing of mRNA that is transcribed from the original type II TGF-ß receptor gene. An important role of this isoform is suggested because the insertion of the human isoform and the murine isoform are localized at the same site and the amino acid sequence of the insertion is highly conserved between these species (76 % identical) (23). It has been shown that $TGF\beta RII_2$ mRNA is expressed in different murine tissues like stomach, intestine, kidney, lung and brain (21). In addition, $TGF\beta RII_2$ mRNA is expressed in several human cell-types like glioblastoma cells, vein endothelial cells, embryonal lung fibroblasts, omental microvascular endothelial cells and lung carcinoma cells (23). However, no data are available about the expression of $TGF\beta RII_2$ mRNA in articular cartilage. Therefore we studied the expression of this isoform in normal articular cartilage of different species. Because TGF-ß responses on articular cartilage seem to be age-related (24-27) and differential TGF-ß responses have been described between normal cartilage and cartilage undergoing repair (28-30) we also studied if the relative expression of $TGF\beta RII_1$ and $TGF\beta RII_2$ is altered during aging or during matrix repair after PG depletion.

MATERIALS AND METHODS

Animals

Male C57Bl/10 mice were used to study age-related differences. Male C57Bl/6 mice were used in experiments in which cartilage depletion was induced. Mice were kept in cages with a wood chip bedding in a room kept at constant temperature. They were fed a standard diet and tap water *ad libitum*.

Isolation of cartilage

Murine articular cartilage was isolated from patellae. Patellae were decalcified in 3.5% EDTA for 4 hours at 4 °C whereafter the whole cartilage layer was stripped off. Because old cartilage is more calcified, decalcification of patellae of old mice (>3 month) was performed over night at 4 °C. In control experiments it was demonstrated that decalcification by EDTA does not affect efficiency of RNA isolation or RT-PCR (data not shown). Bovine cartilage was isolated from metacarpophalangeal joints within 8 hours after death of the animals. Human cartilage was isolated from femoral knee condyles within 18 hours after death of the donor. Only cartilage samples which were histologically defined as normal were used. Isolated human and bovine articular cartilage was immediately frozen in liquid nitrogen.

RNA isolation and RT-PCR

Total RNA was isolated using TRIzol Reagent (Life Technologies). Human and bovine articular cartilage was grinded to powder in a freeze mill and defrosted in TRIzol. Murine articular cartilage was directly after isolation extracted with TRIzol. Before reverse transcription total RNA was treated with DNase (Life Technologies). Reverse transcription was performed with M-MLV Reverse Transcriptase (Life Technologies) using the 3' PCR primer. Taq DNA Polymerase (Life Technologies) was used in the PCR reaction. cDNA was cycled 35 times at 92 °C for 1 min, 55 °C for 1 min and 72 °C for 1 min. The amplified products were separated on an 1.6% agarose gel and visualized by chemoluminescense using the Digoxigenin (DIG) System (Boehringer Mannheim). The PCR primers used for amplification of TGFßRII$_1$ and TGFßRII$_2$ mRNA are described by Suzuki et al (21). Since these primers are located on both sides of the insertion, the PCR

products of TGFßRII$_1$ or TGFßRII$_2$ differ in size (525 bp and 601 bp respectively) and show different mobility on an 1.6% agarose gel.

Induction of mild proteoglycan depletion

Mild PG depletion was induced in 10 weeks old male C57Bl/6 mice by intra-articular injection of papain as described by van der Kraan et al (31). In short, the right knee-joints of the mice were injected once with 6 μl 0.5% papain (Type IV, 15 units/mg, Sigma in 0.03 M L-cysteine.HCL, Sigma). The left control knees were injected with saline. At several points of time after the injection patellae were isolated and used for determination of patellar PG synthesis and RNA extraction for RT-PCR.

Determination of patellar cartilage proteoglycan synthesis

Proteoglycan synthesis was measured *ex vivo*. Whole patellae, with a standard amount of surrounding tissue, were dissected from the knee joints. Patellae were pulse-labeled (2h, 37 °C) with ^{35}S-sulphate (1.1 MBq/ml), After labeling the patellae were washed, fixed, decalcified, punched out of the surrounding tissue, dissolved and counted by liquid scintillation counting as described before (34).

RESULTS

TGFßRII$_1$ and TGFßRII$_2$ mRNA expression in normal cartilage

RT-PCR on isolated RNA from human and murine articular cartilage using TGFßRII specific primers resulted in two PCR products. The sizes of the PCR products corresponded with the expected size of amplified TGFßRII$_1$ mRNA (525 bp) and TGFßRII$_2$ mRNA (601 bp) demonstrating that both TGFßRII $_1$ and TGFßRII$_2$ mRNA are expressed in normal articular cartilage. However, there were striking differences in the relative expression of the two isoforms between species. Murine articular chondrocytes expressed almost equal amounts of TGFßRII$_1$ and TGFßRII$_2$ mRNA while human articular chondrocytes expressed about 3 times more TGFßRII$_1$ mRNA than TGFßRII$_2$ mRNA. When RNA from bovine articular

chondrocytes was used only TGFßRII$_1$ mRNA was detected demonstrating that the expression of the TGFßRII$_2$ isoform is very low or absent (Figure 1).

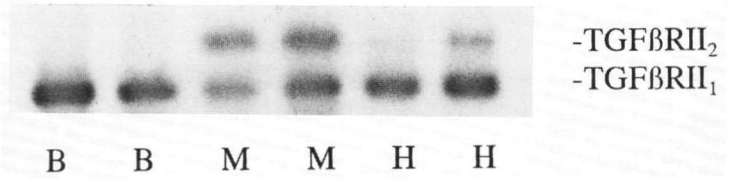

Figure 1 *Expression of TGFßRII$_1$ and TGFßRII$_2$ mRNA in normal bovine (B), murine (M) and human (H) articular cartilage. Total RNA was isolated from normal articular cartilage and RT-PCR was performed. PCR products are separated on a 1.6% agarose gel. Each lane shows the products of RT-PCR using RNA from different donors. RNA from human and bovine cartilage was isolated form individual donors while RNA of murine cartilage was isolated from pooled patellar cartilage of 5 mice.*

TGFßRII$_1$ and TGFßRII$_2$ mRNA expression in cartilage of different age

It has been reported that TGF-ß has different effects on old cartilage compared to young cartilage (24-27). To investigate if these age-related TGF-ß responses are the result of differences in the relative expression of the two isoforms of the TGF-ß type II receptor we determined the relative mRNA expression of TGFßRII$_1$ and TGFßRII$_2$ in murine articular cartilage of different ages. RNA was isolated from patellar cartilage of 3, 6, 12, 18 and 24 months of age whereafter RT-PCR was performed. As shown in figure 2, articular cartilage from mice of all ages express both TGFßRII$_1$ and TGFßRII$_2$ mRNA. No differences could be demonstrated in the relative expression of TGFßRII$_1$ and TGFßRII$_2$ mRNA between 3 months old (young adult) cartilage and cartilage up to 24 months of age (very old).

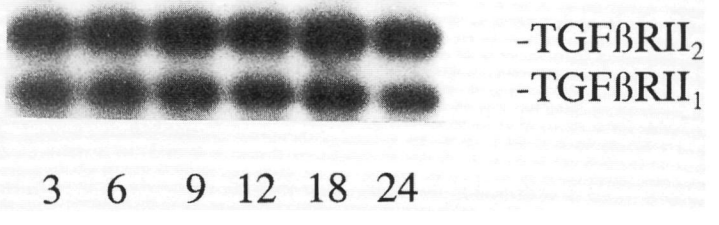

Figure 2 *Expression of TGFßRII$_1$ and TGFßRII$_2$ mRNA in murine articular cartilage between 3 and 24 month of age. RT-PCR was performed using RNA isolated from patellar articular cartilage of different age. RNA was isolated from pooled patellar cartilage of 5 mice. Shown is a representative experiment of three.*

TGFβRII$_1$ and TGFβRII$_2$ mRNA expression in cartilage during a repair phase

Because differential TGF-ß responses have also been described between normal cartilage and cartilage undergoing repair after cartilage injury (11,28,29) we investigated whether the relative expression of TGFßRII$_1$ and TGFßRII$_2$ mRNA is altered during the repair phase after mild PG depletion. A murine cartilage repair model was used in which 0.5% papain was injected in the murine knee joint. Intra-articular injection of papain results in PG depletion (31) and an inhibition of the PG synthesis up to 50% at one day after injection (Figure 3). The PG synthesis is normalized between three and four days after injection whereafter the PG synthesis is supranormal up to 14 days with a maximal stimulation at 7 days after injection.

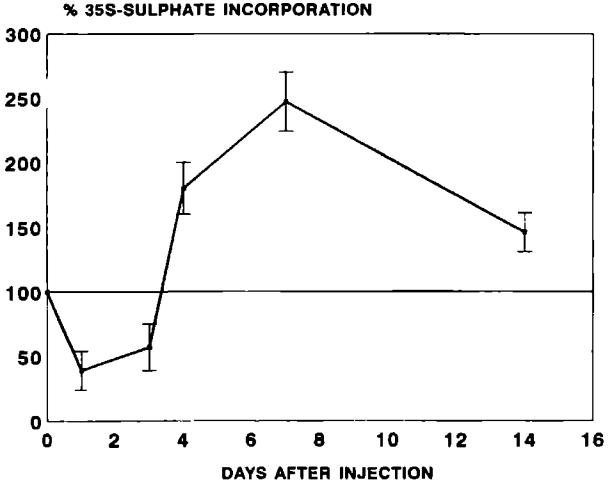

Figure 3 Effect of intra-articular injection of 0.5% papain on the PG synthesis of patellar cartilage. Glycosaminoglycan synthesis was measured ex-vivo by ^{35}S-sulphate incorporation using 5 patellae at each point of time. The results are expressed as percentage of ^{35}S-sulphate incorporation of patellar cartilage from joints which were intra-articular injected with saline. Shown is a representative experiment of three.

The relative TGFßRII$_1$ and TGFßRII $_2$ mRNA expression in cartilage was determined by RT-PCR at different points of time after injection of papain. No differences in the relative mRNA expression of the two isoforms could be demonstrated between normal articular cartilage and cartilage after papain treatment on any point of time (Figure 4).

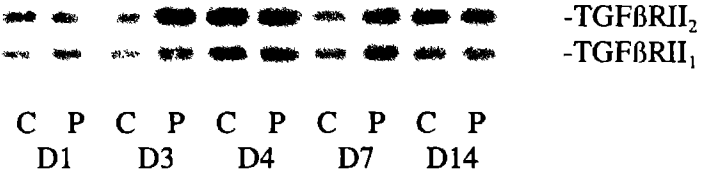

Figure 4 Expression of TGFßRII$_1$ and TGFßRII$_2$ mRNA in patellar cartilage after PG depletion. Murine patellae were dissected at several points of time after intra-articular injection of 0.5% papain (P) or saline (C). RNA was isolated from pooled patellar cartilage of 5 mice. RT-PCR was performed and the PCR products are separated on a 1.6% agarose gel. Shown is a representative experiment of three.

DISCUSSION

Recently a new isoform of the type II TGF-ß receptor was identified (21-23). This new isoform (TGFßRII$_2$) contains an insertion of 25 amino acids in the extracellular domain of the receptor. An important role of this isoform is suggested by the high conservation of this insertion between species. Since the original type II receptor (TGFßRII$_1$) has a low affinity for TGF-ß2 (32,33) it was investigated whether the new isoform was a TGF-ß2 specific receptor. However, binding studies using transfected cells showed that TGFßRII$_2$ also had a much higher affinity for TGF-ß1 than for TGF-ß2 (21,23). These results indicate that the new isoform is not a TGF-ß2 specific receptor. Transfection studies using a TGF-ß resistant cell line which lacks endogenous TGFßRII demonstrated that TGFßRII$_1$ and TGFßRII$_2$ where indistinguishable in their biological functions (23). However, these studies are non physiological and limited to only a few specific TGF-ß responses.

Using RT-PCR we now demonstrated for the first time that both TGFßRII$_1$ and TGFßRII$_2$ mRNA are expressed in normal human and murine articular cartilage. We were not able to detect TGFßRII$_2$ mRNA in bovine articular chondrocytes. Although murine primers were used in these studies we can exclude the possibility that the inability to detect TGFßRII$_2$ mRNA in bovine articular chondrocytes is an artefact due to differences in the primer-annealing sequences. Both TGFßRII$_1$ and TGFßRII$_2$ mRNA were amplified in the same tube, using the same primers and under the same conditions so the isoforms are internal controls for each other. Since TGFßRII$_1$ mRNA could be detected in bovine chondrocytes

we can conclude that bovine articular chondrocytes do not express TGFßRII$_2$ mRNA or express this isoform at a very low level. This implicates that it is unlikely that TGFßRII$_2$ plays an essential role in the metabolism of normal bovine articular cartilage.

TGF-ß responses on articular chondrocytes are shown to be age-related (24-27). For example, articular cartilage from old adult pigs is more sensitive to TGF-ß induced elaboration of extracellular inorganic pyrophosphate (ePPi) than cartilage from juvenile and young adult pigs (26). Young murine articular cartilage appears to be more sensitive to TGF-ß induced stimulation of PG synthesis than old murine articular cartilage (25). Because various TGF-ß responses are age-related we investigated if one of the isoforms is selectively unregulated during aging. However, we demonstrated that the ratio between TGFßRII$_1$ and TGFßRII$_2$ mRNA does not differ in murine articular cartilage between 3 month and 24 month of age. These results suggest that the described differential TGF-ß responses between old and young articular cartilage are not mediated by differences in the relative expression of the two isoforms of TGFßRII.

It has also been demonstrated that TGF-ß has different effects on normal articular cartilage and cartilage during a repair phase. For example, TGF-ß stimulated PG synthesis of human osteoarthritic cartilage while under the same conditions TGF-ß did not have a significant effect on the PG synthesis of normal human cartilage (29). In addition, TGF-ß enhanced the PG synthesis of interleukin-1 (IL-1) treated cartilage but the effects of TGF-ß on normal cartilage PG synthesis were minimal(28,30). We investigated the relative expression of TGFßRII$_1$ and TGFßRII$_2$ mRNA in the repair phase after mild PG depletion. Mild PG depletion in murine articular cartilage was induced by injection of 0.5% papain in the murine knee joint. No differences in the relative expression of TGFßRII$_1$ and TGFßRII$_2$ mRNA could be demonstrated between normal cartilage and cartilage undergoing repair after mild PG depletion. This indicates that the supranormal PG synthesis after PG depletion by papain is not mediated by selective upregulation of mRNA of one of the two isoforms. This also suggests that the described differential TGF-ß responses between normal cartilage and cartilage undergoing repair (28-30) are not the result of differences in the relative expression of TGFßRII$_1$ and TGFßRII$_2$ mRNA.

In summary, this study demonstrates that the newly identified isoform of the type II TGF-ß receptor ($TGF\beta RII_2$) is expressed in normal human and murine articular cartilage. $TGF\beta RII_2$ mRNA was not detectable in bovine articular cartilage indicating that it is unlikely that $TGF\beta RII_2$ plays an important role in the physiology of normal bovine articular cartilage. No differences in the relative mRNA expression of the two isoforms of the type II TGF-ß receptor could be demonstrated in murine cartilage during aging or during the repair phase after mild PG depletion indicating that it is unlikely that age-related TGF-ß responses and differential TGF-ß responses between normal cartilage and cartilage undergoing repair are the result of differences in the relative expression of the two TGFßRII isoforms.

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CHAPTER 4

BMP-2 AND TGF-β1 BOTH STIMULATE ARTICULAR CARTILAGE PROTEOGLYCAN SYNTHESIS AND INDUCE CHONDROGENESIS *IN VIVO*, BUT SHOW QUANTITATIVE AND QUALITATIVE DIFFERENCES

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ABSTRACT

The related molecules bone morphogenetic protein-2 (BMP-2) and transforming growth factor beta-1 (TGF-ß1) have both been shown to stimulate chondrocyte proteoglycan (PG) synthesis *in vitro*. The objective of this study was to determine the *in vivo* effects of these factors on articular cartilage PG metabolism. Several dosages of BMP-2 or TGF-ß1 were injected into the murine knee joint, once or repeatedly. Patellar cartilage PG synthesis was measured by ^{35}S-sulfate incorporation and PG content was analyzed by measuring safranin O staining intensity on histologic sections. A single injection of 200 ng BMP-2 induced a much earlier and more impressive stimulation of articular cartilage PG synthesis, than 200 ng TGF-ß1. RT-PCR revealed that both factors upregulated mRNA of aggrecan more than that of biglycan and decorin. However, 21 days after a single injection of 200 ng TGF-ß1 PG synthesis still was significantly increased, while stimulation by BMP-2 only lasted for 3 to 4 days. Stimulation by BMP-2 could be prolonged to at least 2 weeks by triple injections of 200 ng each, at alternate days. Remarkably, even after this intense exposure to BMP-2, stimulation of PG synthesis was not reflected in long-lasting enhancement of PG content of articular cartilage. In contrast, even a single injection with 200 ng of TGF-ß1 induced prolonged enhancement of PG content.

After repeated injections, both BMP-2 and TGF-ß1 induced chondrogenesis at specific sites. "Chondrophytes" induced by BMP-2 were found predominantly in the region where the growth plates meet the joint space, while those triggered by TGF-ß originated from the periosteum also at sites remote from the growth plates. We conclude that BMP-2 and TGF-ß stimulate PG synthesis and PG content with different kinetics, and that these factors have different chondro-inductive properties.

INTRODUCTION

Loss of proteoglycans (PGs) from articular cartilage is a feature of several joint diseases. Depletion of these highly sulfated and hydrated molecules decreases the resistance of cartilage to mechanical forces and to extended enzymatic degradation and could therefore be the first step in cartilage degeneration. Factors which are able to stimulate PG synthesis and to accelerate replenishment of PGs in depleted cartilage could be of significant therapeutic value. Transforming growth factor beta-1 TGF-ß1) and bone morphogenetic protein-2 (BMP-2) have been shown to stimulate chondrocyte PG synthesis *in vitro* (1-5). Therefore, they are possible candidates in the search for factors that improve restoration of articular cartilage.

Transforming growth factors (TGF-ßs) and bone morphogenetic proteins (BMPs) belong to the TGF-ß superfamily (6,7). This superfamily consists of dimeric molecules of which each monomer contains at least seven conserved cysteine residues (8). The proteins signal by serine/threonine kinases (6,9). In earlier studies (10) we showed that intra-articular injection of 200 ng TGF-ß1 into the knee joint of C57Bl/6 mice induces long-lasting stimulation of patellar cartilage PG synthesis. However, we also found that multiple injection induces fibrosis (thickening of synovium and ligaments) and formation of "chondrophytes", originating from the periosteum, and developing into osteophytes. In the present study we tried to identify dose regimens which might preserve the anabolic effect on cartilage without pronounced side effects on synovial tissue and periosteal tissue. Moreover, we made a comparison with BMP-2. The latter factor belongs to the BMP family and has been shown to possess strong chondrocyte PG synthesis stimulatory activity, *in vitro* (3,4), whereas information on *in vivo* effects on cartilage metabolism is lacking. It was found that both factors stimulate chondrocyte PG synthesis, but with markedly different kinetic profiles. Moreover, both factors induced chondrophytes, but at different, characteristic, regions of the joint.

MATERIALS AND METHODS

Animals

Male C57Bl/6 mice aged 12 weeks were used. They were fed a standard diet and tap water *ad libitum*.

Growth Factors

Recombinant human BMP-2, rhTGF-ß1, and rhTGF-ß2 were kindly provided by Genetics Institute Inc (Cambridge, MA, USA), Genentech Inc (San Francisco, CA, USA), and Novartis (Basel, Switzerland), respectively.

Intra-articular injections

BMP-2 and TGF-ßs were dissolved in saline + 0.1% ultrapure bovine serum albumin (Sigma, St Louis, MO, USA). Six μl volumes were injected into the joint cavity of the right knee. BMP-2 and TGF-ß were administered in 2, 20, 200, or 400 ng dosi. Simultaneously, the contralateral joint received an equal volume of vehicle (saline + 0.1% bovine serum albumin). Each joint was injected once or three times at alternate days.

Histology

Whole knee joints were dissected and processed as previously described (11). Semiserial frontal sections were stained by hematoxylin/eosin or safranin O/fast green for examination of cells and cartilage matrix, respectively. For autoradiographic analysis of ^{35}S-sulfate incorporation (11), radiolabeled sulfate (75μCi) was injected intraperitoneally 6 hours before dissection of the knee joints. After histologic processing, 6-μm sections were prepared and mounted on gelatin-coated slides. These were dipped in K_5 emulsion (Ilford, Basildon, Essex, UK) and exposed for 3 to 5 weeks. After this period, the slides were developed and stained with hematoxylin and eosin.

Determination of patellar cartilage proteoglycan synthesis

Proteoglycan synthesis was measured ex vivo according to the method of van den Berg et al. (12). Patellae with a standard amount of surrounding tissue were dissected from the knee joints. Patellae were then pulse-labeled (2 hours, at 37°C) with ^{35}S-sulfate. Subsequently, they were washed, fixated in ethanol, and decalcified in formic acid. After decalcification the entire cartilage was stripped off, dissolved in lumasolve and radioactivity was measured by liquid scintillation counting. In some experiments a piece of 0.2 mm^2 was punched out of the patellar cartilage for study of the homogeneity (central part relative to peripheral part) of growth factor effects (13).

Determination of patellar cartilage proteoglycan content

Articular cartilage PG content is reflected in safranin O staining intensity in histological sections. This was measured using an automated image analysis system as described before (14). Fast green staining was neutralized by use of a green filter. Optical density was examined in the non-calcified cartilage. Measurements were corrected for chondrocyte lacunae and for background measured in PG depleted cartilage in which no red stain was visible any more.

RNA extraction and RT-PCR

Patellae were dissected and immediately decalcified in 3.5% EDTA for 4 hours at 4°C. Following decalcification the complete articular cartilage layer was stripped from the underlying bone. The isolated cartilage was instantly put in TRIzol reagent (Life Technologies) for RNA extraction. In control experiments it was shown that this procedure did not affect the RNA isolation or RT-PCR negatively (15). RNA was directly extracted from cartilage, without homogenization of tissue. Cartilage of 10 patellae was pooled. Before reverse transcription the isolated RNA was treated with DNAse 1 (Life Technologies). The reverse transcription reaction was performed with moloney-murine leukemia virus (M-MLV) reverse transcriptase (Life Technologies), using an oligo(dT)$_{15}$ primer (Eurogentec, Liege, Belgium). Amplification of DNA was accomplished by using Taq DNA polymerase (Life Technologies) up to a cycle number of 40. To estimate the relative mRNA levels, 5 μl samples were taken at increasing cycle numbers. The PCR products were electrophorised in 1.6% agarose gels containing ethidium bromide. The cycle number at which the product was first detected on the gel was

taken as a measure for the amount of specific mRNA present in the originally isolated RNA. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) mRNA levels were used as an internal control. This method was validated by van Meurs et al. (15). All RT-PCR reactions were performed in duplicate.The following primers were used in the amplification reactions.
GAPDH: 5'AACTCCCTCAAGATTGTCAGCA-3'(upper),5'-TCCACCACCCTGT TGCTGTA-3', resulting in a 553 bp product. Biglycan: 5'-AGAAGGCCTTTAGC CCTCCTG-3' (upper), 5'-ACTTTGCGGATACGGTTGTC-3' (130 bp product). Aggrecan primers were used as described by Grover and Roughly (16) resulting in a 501 bp product, while decorin primers were used according to Asundi and Dreher (17)(400 bp). Primers detecting murine collagen type X had the following sequences: 5'-ATACCCTTTCTGCTGCTAATGTTCTTGACC-3' (upper), 5'-TGATATTCCTGGTGGTCCTGGCAAC-3' (lower), resulting in a 387 bp product.

Statistical analysis

Differences between experimental groups were tested using the Student's t-test. Differences were considered significant if $P < 0.05$.

RESULTS

Stimulation of articular cartilage PG synthesis

Dose-response studies over a one-week period showed that one intra-articular injection of 200 ng BMP-2 increased patellar cartilage PG synthesis up to three times the normal level within 2 days (Figure 1a). Thereafter PG synthesis declined and reached nearly basal levels at day 5 after injection. The stimulation of PG synthesis could not be increased beyond the level reached with 200 ng by using a higher BMP-2 dosage (400 ng, not shown). One injection with 20 ng BMP-2 stimulated PG synthesis to 150% of basal level, but this stimulation was lost within 3 days, while 2 ng was without significant effects. A single injection of TGF-ß1 also stimulated PG synthesis but the maximum level reached was lower; only twice the normal synthesis rate (Figure 1b). Moreover, this level was reached later (after four days) and stimulation of PG synthesis lasted longer as compared to BMP-2 injection. Significant stimulation was found up to one month after injection of 200 ng TGF-ß (Figure 2). Even the 2 ng TGF-ß1 dose stimulated PG synthesis for at least 7 days. Triple injections did not further increase or prolong TGF-ß1-induced

stimulation of PG synthesis (Figure 3). In contrast, prolonged stimulation was found after triple injections of 200 ng BMP-2. Autoradiography showed that ^{35}S-sulfate incorporation was stimulated homogeneously throughout the articular cartilage both after BMP-2 and TGF-ß1 injections (Figure 4). Using scintillation counting we confirmed that effects on ^{35}S-incorporation were similar in the central and peripheral parts of patellar cartilage (data not shown). Effects of TGF-ß1 and TGF-ß2 on proteoglycan synthesis appeared to be comparable (data not shown)

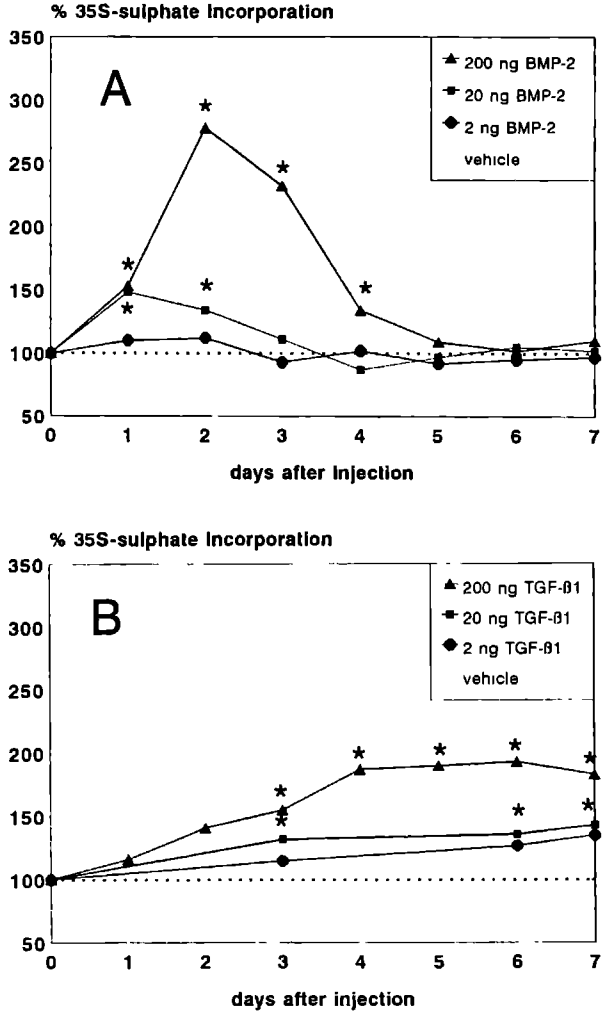

*Figure 1 Dose-dependent stimulation of patellar cartilage PG synthesis after a single injection of BMP-2 (A) or TGF-ß1 (B). Even the lowest dose of TGF-ß1 still had significant effect ($p < 0.05$) at day 7. ^{35}S-sulfate incorporation is expressed as % of the vehicle-injected control. Each value represents the mean of at least 12 animals. * = significant increase ($p < 0.05$).*

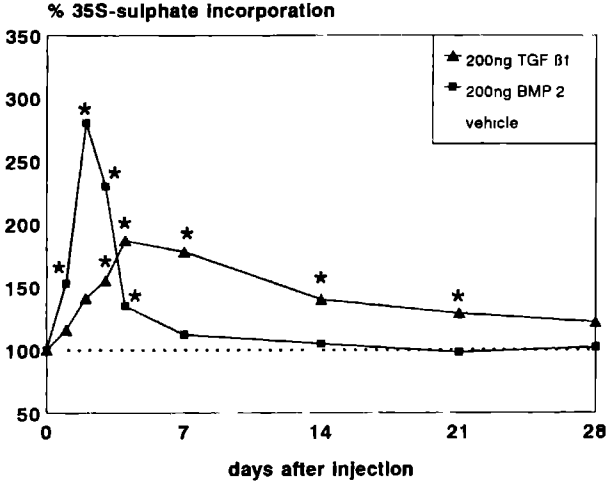

*Figure 2 Duration of stimulation of PG synthesis after a single injection of 200 ng BMP-2 or TGF-β1 The effect of BMP-2 was lost after 5 days, while TGF-β1 still had significant effect ($p < 0.05$) on day 21 ^{35}S-sulfate incorporation is expressed as % of the vehicle-injected control. Each value represents the mean of at least 12 animals *= significant increase ($p < 0.05$)*

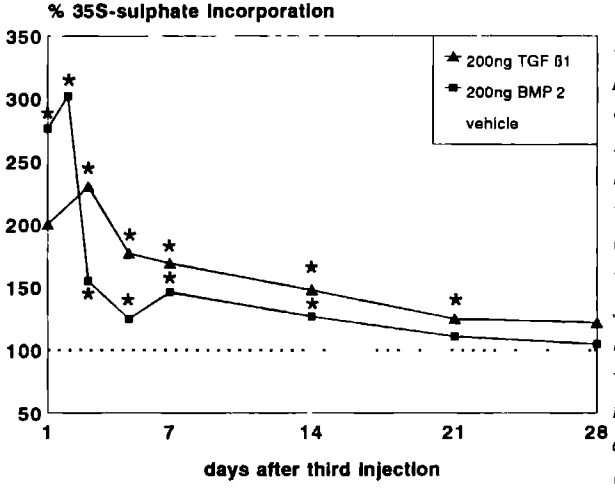

*Figure 3 Duration of stimulation patellar cartilage PG synthesis after triple injections of 200 ng BMP-2 or TGF-β1 By giving multiple injections the effect of BMP-2 was prolonged to 14 days ($p < 0.05$), while stimulation by TGF-β1 was comparable to the effect of a single injection ^{35}S-sulfate incorporation is expressed as % of the vehicle-injected control Each value represents the mean of at least 12 animals *=significant increase ($p < 0.05$).*

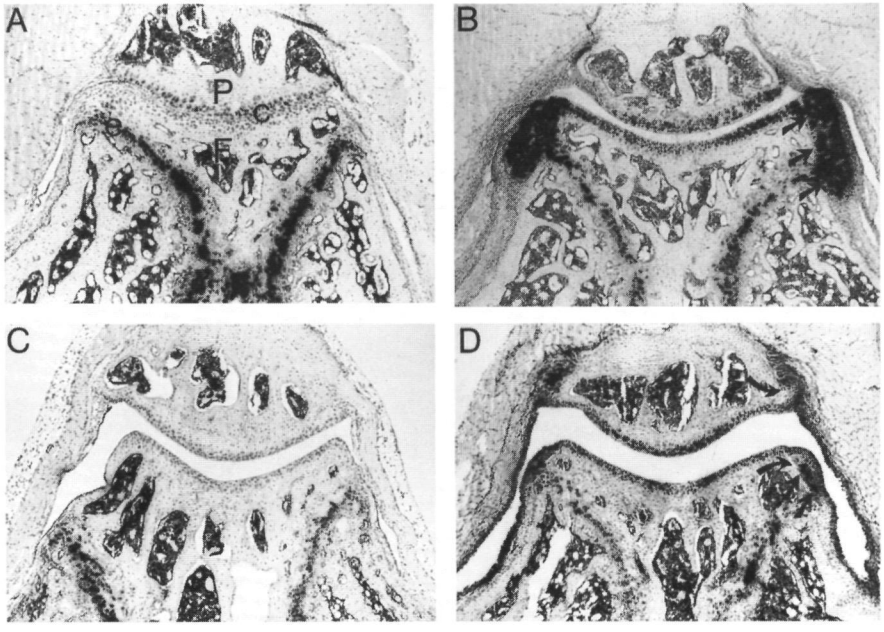

Figure 4 *Autoradiographs showing in vivo ^{35}S-sulfate incorporation in cartilage and chondrophytes (arrows) after BMP-2 or TGF-β triple injections (200 ng each); hematoxylin/eosin stained frontal sections of murine knee joint.* ***A)*** *vehicle-injected control joint of B,* ***B)*** *contralateral joint of the same animal as A at day 4 after the last BMP-2 injection,* ***C)*** *vehicle-injected control joint of D,* ***D)*** *contralateral joint of the same animal as C at day 4 after the last TGF-β injection. p = patella, f = femur, c = articular cartilage, e = epiphyseal cartilage (original magnification x 100)*

Upregulation of mRNA of different PG types and of collagen X

Overall PG synthesis can be determined by measurement of ^{35}S-sulfate incorporation, but this reflects mainly aggrecan synthesis. To get insight in potential effects of BMP-2 and TGF-ß1 on mRNA expression of different PG types we used RT-PCR. Messenger RNA levels were determined of three abundant PG types in articular cartilage; aggrecan, biglycan and decorin. At the time point of highest PG synthesis after the last of three BMP-2 injections, day 2, the mRNA levels of aggrecan, biglycan, and decorin were increased to 8, 4 and 2 times basal values, respectively. A single injection of 200 ng BMP-2 had less, but still signifi-

cant effect; At day 2 after one BMP-2 injection, the amount of mRNA of decorin was unchanged, while that of aggrecan and biglycan had increased to 4 times basal values In contrast, TGF-ß injections induced upregulation of only aggrecan mRNA (2-4-fold) one day after the last of three 200 ng injections. However, this was already normalized one day later. Interestingly, the amount of mRNA encoding for collagen type X, indicative for chondrocyte hypertrophy, was unchanged after TGF-ß exposure, but highly upregulated after BMP-2 injections (± 16 times normal values at day 2 after third injection).

Elevation of articular cartilage PG content

In order to elucidate whether the stimulation of PG synthesis was reflected in an increase in articular cartilage PG content, intensity of safranin O staining in histological sections was measured. BMP-2 injections (3 x 200 ng) appeared to induce only a small, short-lived increase of patellar cartilage PG content (table 1), while TGF-ß1 injections (3 x 200 ng) induced prolonged enhancement of the PG content, lasting at least 2 weeks after the third injection. A single injection of 200 ng TGF-ß1 was almost as effective in this respect as triple injections. Effects of TGF-ß1 and TGF-ß2 on PG content appeared to be comparable (data not shown).

Table 1 *Effect of intra-articular injections on patellar cartilage proteoglycan content*

Injected substance	days*	% increase of proteoglycan content§	
1 x TGF-ß	7	16 ± 7#	
1 x TGF-ß	14	17 ± 8#	
3 x TGF-ß	1	27 ± 13#	
3 x TGF-ß	4	25 ± 11#	
3 x TGF-ß	7	22 ± 8#	
3 x TGF-ß	14	15 ± 6#	
3 x BMP-2	1	26 ± 15#	
3 x BMP-2	2	10 ± 10	not significant
3 x BMP-2	4	9 ± 7	not significant

* *days after the last injection, in the triple injection protocol three injections (200 ng each) were given, at alternate days, meaning that in this protocol day 1 after the last injection = 5 days from start*

§ *safranin O staining intensity of histological sections measured using an automated image analyzer Staining intensity of patella cartilage in vehicle-injected knee joints was stated 100% Each experimental group consisted of at least 10 mice, and of each joint at least 4 sections were analyzed # $p < 0.01$, significantly different from vehicle-injected joints*

Induction of osteophytes

Histologic sections of knee joints demonstrated that intra-articular injection of TGF-ß and BMP-2 resulted in formation of new chondroid tissues (Figure 5). Interestingly, these new structures show different characteristics. BMP-2 induced outgrowth of epiphyseal cartilage, especially in the femur, at the level where the growth plate approaches the articular cartilage of the patellar groove. This chondrogenic activity appeared to be restricted to those regions in the growth plate adjacent to the joint space, in close contact with the periosteum. Autoradiography (Figure 4) showed no effect of BMP-2 on ^{35}S-sulphate incorporation in the epiphyseal cartilage, indicating local activation of only the area where the growth plate meets the joint space. Besides in the described regions, BMP-2 did not induce notable development of chondroid tissue. In contrast, TGF-ß induced chondrogenesis originating from the periosteum with apparently no need for direct contact with epiphyseal cartilage. The TGF-ß-induced 'chondrophytes' developed close to the margins of articular cartilage, at the insertion sites of ligaments, and at the base of menisci. They were found also on the patella, which has no growth plate in mice, and in superficial frontal sections of the knee, which did not include epiphyseal cartilage (Figure 5). Chondro-inductive effects of TGF-ß1 and TGF-ß2 appeared to be comparable (not shown). Injection of TGF-ß1 and BMP-2 together showed clear synergism of these two factors in chondrogenesis (Figure 5). At later time points chondrophytes lost their PGs (no safranin O staining) and developed into osteophytes, containing bone marrow (Figure 5). Mature osteophytes induced by BMP-2 and TGF-ß looked very much alike, except for their localization.

In search for treatments that stimulate articular cartilage PG synthesis for at least a week, without inducing chondrophytes, dose-response studies were performed (table 2). These studies indicated that in the triple injection protocol chondro-induction and longterm stimulation of PG synthesis by TGF-ß or BMP-2 could not be uncoupled. TGF-ß was more potent than BMP-2 in both effects. The only protocol that met the above-mentioned requirements, was a single injection of TGF-ß. Even the 200 ng dose did not induce chondrophytes in this protocol, and as has been shown in figure 1B a single injection of all tested TGF-ß dosages (2, 20, 200 ng) induced long-term stimulation of PG synthesis.

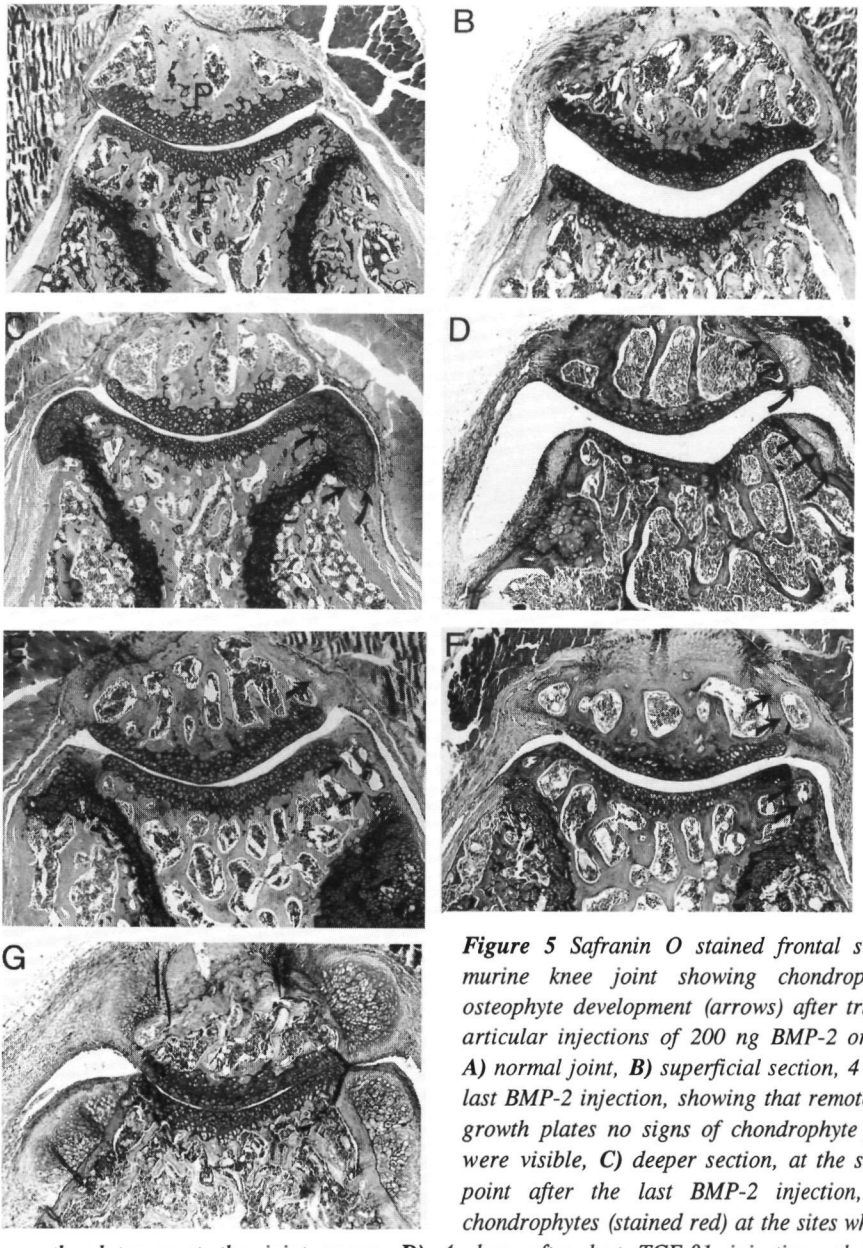

Figure 5 *Safranin O stained frontal sections of murine knee joint showing chondrophyte and osteophyte development (arrows) after triple intra-articular injections of 200 ng BMP-2 or TGF-β1.* ***A)*** *normal joint,* ***B)*** *superficial section, 4 days after last BMP-2 injection, showing that remote from the growth plates no signs of chondrophyte formation were visible,* ***C)*** *deeper section, at the same time-point after the last BMP-2 injection, showing chondrophytes (stained red) at the sites where growth plates meet the joint space,* ***D)*** *4 days after last TGF-β1 injection, chondrophyte development more independent of growth plate (also chondrophytes on the patella) was found; also note synovial hyperplasia,* ***E)*** *21 days after last BMP-2 injection chondrophytes had developed into osteophytes, not stained with safranin O and containing bone marrow,* ***F)*** *21 days after the last TGF-β1 injection, osteophytes had developed that looked very similar compared to those induced by BMP-2,* ***G)*** *synergism of TGF-β1 and BMP-2 chondroinductive (and fibrotic) actions, 4 days after the last injection of BMP-2 + TGF-β1 . (original magnification x 100)*

Table 2 Correlation between long-term stimulation of PG synthesis and induction of chondrophytes by BMP-2 and TGF-β.

injected substance	chondrophytes	stimulation of PG synthesis (> 1 week)
1 x 200 ng TGF-ß	-	+
3 x 200 ng TGF-ß	+	+
3 x 20 ng TGF-ß	+	+
3 x 2 ng TGF-ß	-	-
1 x 200 ng BMP-2	-	-
3 x 200 ng BMP-2	+	+
3 x 20 ng BMP-2	-	-

Induction of fibrosis

TGF-ß was much more potent than BMP-2 in inducing fibrosis. Triple injections with 200 ng TGF-ß increased the amount of fibroblasts in the synovial sublining, and also the diameter of collateral ligaments clearly increased (Figure 6). BMP-2 injections had only very little effect in this respect, but in some cases a granulous tissue developed at the extraarticular side of collateral ligaments. Similar structures, but larger, were found after injections of BMP-2 + TGF-ß1.

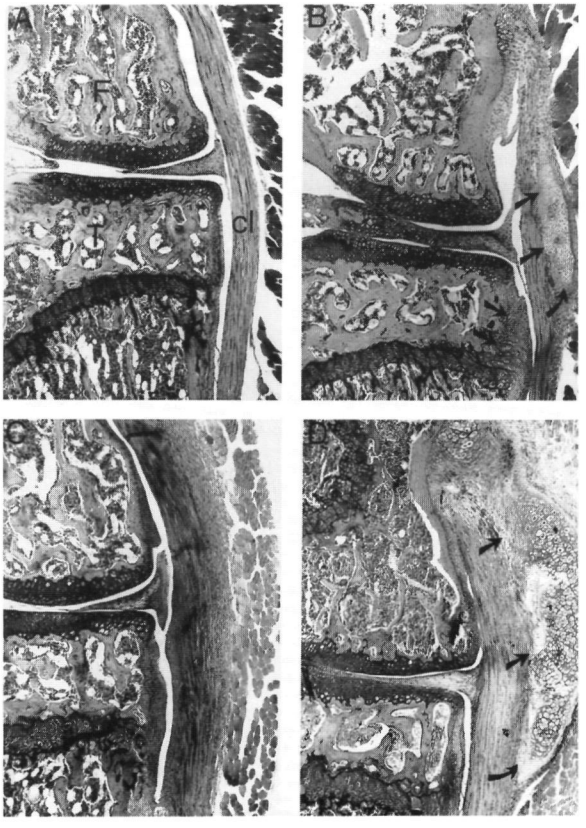

Figure 6 *Safranin O stained frontal sections of murine knee joint showing effects on collateral ligaments induced by triple intra-articular injections of 200 ng BMP-2 or TGF-ß1.* ***A)*** *normal joint* ***B)*** *4 days after the last BMP-2 injection there is no fibrosis of ligaments, but in some cases a new granulous structure, hardly stained, has developed at the extraarticular side of the ligament, and a chondrophyte is present at the end of the growth plate (arrows)* ***C)*** *4 days after the last TGF-ß1 injection the collateral ligaments are thicker and in some cases they stain red instead of green, indicating enhanced PG content, presumably a sign of chondrogenesis* ***D)*** *4 days after the last coinjection of TGF-ß1 and BMP-2, with large chondrophyte at the extra-articular side of the ligament (arrows). (original magnification x 100)*

DISCUSSION

In vitro, members of the BMP family like BMP-2, BMP-4, BMP-7, and BMP-9 have been demonstrated to stimulate articular chondrocyte PG synthesis (3,4,18,19), but until now no *in vivo* data were available. We reported earlier that TGF-ß1 is a potent stimulator of articular cartilage PG synthesis and content *in vivo* (10). In the present study, we focused on the comparison of effects of intra-articular injections of TGF-ß1 and BMP-2 on articular cartilage PG synthesis and content *in vivo*.

BMP-2, like TGF-ß1, appeared to be a potent stimulator of articular cartilage PG synthesis *in vivo*. PG synthesis was stimulated homogeneously throughout the articular cartilage. Interestingly, stimulation of PG synthesis by BMP-2 and TGF-ß1 showed different kinetics. Stimulation of PG synthesis by BMP-2 was much earlier and stronger, but also of shorter duration than stimulation by TGF-ß. The retarded stimulation of PG synthesis by TGF-ß could indicate that the first event is

production of a second mediator, or a change in chondrocyte phenotype. An explanation of the prolonged stimulation of PG synthesis seen after TGF-ß injection could be TGF-ß autoinduction (20), but also changes in mechanical forces on articular cartilage due to TGF-ß-induced fibrosis and chondrophyte development could be responsible. In contrast to the long-term disturbance of overall PG synthesis, TGF-ß induced only slight, transient changes in mRNA levels of aggrecan and did not change message of the two small PGs. Thus, on the mRNA level we found no indication of changes in the balance between large and small PGs during TGF-ß-induced PG overproduction. Substantial shifts in favour of the smaller PG classes were described in the literature, but this always concerned TGF-ß effects on other cell types and *in vitro* (21-23). Reported TGF-ß effects on human articular chondrocyte PG gene expression *in vitro* (24,25) are much smaller (biglycan x 2, decorin x 0.5, aggrecan x 4). This is more in line with our findings in murine articular cartilage *in vivo*, especially if we take into account that with RT-PCR it is not easy to pick up a shift of one cycle. BMP-2 effects on PG message were larger than those of TGF-ß, and resulted in a shift in favour of aggrecan. Because of the short duration of BMP-2 effects on PG synthesis, and content, we suppose this will not have too much influence on the composition of the cartilage extracellular matrix. BMP-2 also strongly stimulated collagen type X mRNA expression. As type X collagen is synthesized primarily by hypertrophic chondrocytes (26,27), this finding indicates that BMP-2 may be involved in induction of the hypertrophic phenotype.

Stimulation of PG synthesis was reflected in increased PG content after TGF-ß, but not after BMP-2 injections. This might be caused by the short duration of BMP-2 induced stimulation of PG synthesis, but also TGF-ß could promote PG binding to matrix or chondrocytes by inducing HA production (28) or by stimulation of integrin expression on chondrocytes (29). Moreover, TGF-ß might enhance PG content by suppressing PG degradation (1,24,30). However, also members of the BMP family seem to have, at least *in vitro*, the potential to inhibit PG breakdown (19,31).

Apart from its strong effects on cartilage PG metabolism, TGF-ß was also much more potent than BMP-2 in induction of fibrosis. This was seen in the synovium, but also ligaments increased in diameter after TGF-ß injections. The difference in fibrogenic properties of BMP-2 and TGF-ß1 we showed is in line with a study that compared BMP-7 and TGF-ß1 effects on fibroblast proliferation and matrix synthesis (32). Another periarticular change we observed was the induction of chondrophytes, which eventually developed into osteophytes. Intra-articular

injections of BMP-2 resulted in the formation of new chondroid tissue, especially in the femoro-patellar area. The ability of BMP-2 to induce the formation of new cartilage and bone has been demonstrated before, using the rat ectopic bone formation assay (7,33). Other members of the BMP family, like BMP-3, BMP-4, BMP-5, and BMP-7 are also able to induce new cartilage and bone *in vivo* (7,33,34). As we demonstrated before, intra-articular injections of TGF-ß1 induced chondrophytes which develop into osteophytes (10). Interestingly, the BMP-2-induced chondrophytes are quite different as compared to those induced by TGF-ß. The BMP-2-induced chondrophytes are always growing in close contact with the area where growth plates meet the joint space, while those induced by TGF-ß seem to develop more independently of these sites. Also the observation that the most pronounced TGF-ß-induced chondrophyte development was on the patella (lacking a growth plate), while after BMP-2 injections chondrophytes were rarely seen on the patella, but predominantly on the femur, points in this direction. Autoradiography did not show BMP-induced activation, at the level of proteoglycan synthesis, of the growth plates themselves. This indicates that the chondroid tissue had originated from the periosteum, or from the surface of the growth plate adjacent to the joint space. Several studies performed in other systems also show that BMP-2 and TGF-ß act different in inducing cartilage and bone production (35-37). This could indicate that BMP-2 and TGF-ß may act by different mechanisms, or that because of variable receptor expression they activate different cell populations. Additional evidence for the existence of differential activation pathways is the synergism in chondrophyte induction that we found after injecting BMP-2 and TGF-ß into the same joint. Because of the characteristic appearance and localization of early BMP-2-induced chondrophytes, we can speculate about the relative contribution of BMP and TGF-ß in osteophyte induction during natural processes in mice. From the appearance of arthritis-induced chondrophytes we conclude that there is no physiological role for BMP in this process. Moreover, the localization of osteophytes in naturally occurring and experimental osteoarthritis in mice also is more similar to that induced by TGF-ß than BMP-2 injections, but we do not know what early chondrophytes in these conditions look like.

A single injection of 200 ng TGF-ß appeared to induce long-term enhancement of articular cartilage PG synthesis and content, with only moderate fibrosis and no chondrophytes. For induction of chondrophyte formation repeated injections were needed. Three injections of a 10 times lower dose still induced chondrophyte formation. BMP-2 was less potent also in this respect, because three injections of 20 ng BMP-2 appeared to have no chondroinductive capacity. In the triple injection

protocol we did not succeed in finding dosages that had significant effects on cartilage PG metabolism, without inducing chondrophytes. There is evidence that periosteum of rodents is more responsive to chondrophyte-inducing factors as compared to primates (38,39), indicating that BMP- or TGF-ß-induced formation of chondrophytes and osteophytes might be less of a problem in humans.

In summary, this study demonstrates that local administration of BMP-2 or TGF-ß into normal joints stimulates articular cartilage PG synthesis. TGF-ß has the highest impact, because of its long-lasting enhancement of both PG synthesis and PG content, and seems to be the most promising factor for replenishment of PG in depleted cartilage in pathological conditions. However, formation of chondrophytes might limit the therapeutic applications of TGF-ß and BMP-2.

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CHAPTER 5

BONE MORPHOGENETIC PROTEIN-2 STIMULATES ARTICULAR CARTILAGE PROTEOGLYCAN SYNTHESIS IN VIVO BUT DOES NOT COUNTERACT INTERLEUKIN-1α EFFECTS ON PROTEOGLYCAN SYNTHESIS AND CONTENT

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ABSTRACT

Objective. To study effect of bone morphogenetic protein-2 (BMP-2) on articular cartilage proteoglycan (PG) synthesis in vivo and to investigate if BMP-2 is able to counteract the effects of interleukin-1 (IL-1) on articular cartilage PG synthesis and content.

Methods. BMP-2 alone or in combination with IL-1α was injected into murine knee joints. PG synthesis was measured by ^{35}S-sulfate incorporation using an ex vivo method or autoradiography. Cartilage PG content was analyzed by measuring safranin O staining intensity on histologic sections.

Results. BMP-2 appeared to be a potent stimulator of articular cartilage PG synthesis in vivo. However, BMP-2 was not able to counteract the deleterious effects of IL-1α on articular cartilage PG synthesis and content. In addition, intra-articular injections of BMP-2 induced chondrophytes.

Conclusion. Although BMP-2 is a very potent stimulator of cartilage PG synthesis in vivo, the therapeutic applications of BMP-2 are limited due to the inability of BMP-2 to counteract the effects of IL-1 and the induction of chondrophytes.

INTRODUCTION

Rheumatoid arthritis is a disease characterized by chronic inflammation of the joints. The disease causes cartilage degradation which results in the loss of joint function. An early event in the process of cartilage degradation is depletion of proteoglycans (PG) from articular cartilage. Interleukin 1 (IL-1) is an important mediator in this process. IL-1 is able to enhance PG degradation and to suppress PG synthesis (1-4). Moreover, studies in which IL-1 was neutralized during experimental arthritides demonstrated that IL-1 is directly involved in the inhibition of articular cartilage PG synthesis (4-7).

Factors which are able to counteract the effects of IL-1 on chondrocyte metabolism or which are able to stimulate the replenishment of proteoglycans in the depleted matrix could be of significant therapeutic value. In this respect bone morphogenetic proteins (BMPs) seem promising. BMPs belong to the transforming growth factor ß (TGF-ß) superfamily (8,9). This superfamily consist of dimeric molecules, each of which contains 7 conserved cysteine residues (10). The proteins signal by serine/threonine kinases (8,11). One of the members of the BMP family with potential therapeutic value is bone morphogenetic protein-2 (BMP-2). BMP-2 has been demonstrated to be a potent stimulator of chondrocyte metabolism and differentiation (12-15). BMP-2 is a potent stimulator of PG synthesis of articular cartilage explants in vitro (12) but until now no in vivo data about the effects of BMP-2 on articular cartilage PG synthesis have been available. Neither have any data been published about the ability of BMP-2 to counteract the effects of IL-1 on articular cartilage. We therefore studied the effect of BMP-2 on murine articular cartilage PG synthesis in vivo and investigated the ability of BMP-2 to counteract the effects of IL-1 on articular cartilage PG synthesis and content.

MATERIALS AND METHODS

Animals

Male C57Bl/6 mice between 8 and 12 weeks of age were used. They were fed a standard diet and tap water ad libitum.

Growth factors and cytokines

Recombinant murine IL-1α (0.48 mg/ml in PBS pH 7.4) and recombinant human TGF-ß1 (0.1 mg/ml in 20 mM NaOAc pH 5.0) were kindly provided by respectively Pfizer Central Research (Groton, CT, USA) and Genentech Inc (South San Francisco, CA, USA). Recombinant human BMP-2 (2.27 mg/ml in 0.5M Arginine, 10 mM Histidine pH 6.5) was supplied by Genetics Institute Inc (Cambridge, MA, USA). To prevent loss of protein due to adherence to plastic only siliconized tubes and tips were used.

Intra-articular injections

To study the effect of BMP-2 on articular cartilage PG synthesis in vivo 6 μl physiological saline + 0.1 % bovine serum albumin including rhBMP-2 (2-1000 ng) was injected into the joint cavity of the right knee. The ability of BMP-2 to counteract the effects of IL-1 on articular cartilage PG synthesis and content was studied by injecting IL-1α (10 ng), either alone or in the presence of BMP-2 (200-1000 ng). The dose of 10 ng IL-1α has been demonstrated to suppress articular cartilage PG synthesis after a single injection and to induce significant PG depletion in multiple injection protocols (2). One single injection or 3 injections were given on alternate days. Since we have previously demonstrated that TGF-ß1 is able to counteract the effects of IL-1 on articular cartilage PG synthesis and content (16,17) we used coinjections of IL-1α (10 ng) and TGF-ß1 (200 ng) as positive controls.

Histology

Whole knee joints were dissected and fixed for 7 days in phosphate-buffered formalin. The fixed knee joints were decalcified (5% formic acid) and dehydrated by an automated tissue processing apparatus (VIP, Miles Scientific, Naperville, IL, USA). After embedding in paraffin wax semiserial frontal knee sections (6 μm) were prepared and mounted on gelatin-coated slides. Paraffin was removed by xylol and ethanol and sections were stained with safranin O and fast green (18). Safranin O staining, a semiquantitative marker of PG depletion, was measured using an automated image analysis system (VIDAS, Kontron Electronics, Munich, Germany) (19). Fast green staining was neutralized by use of a green filter. Optical density was examined in the noncalcified cartilage of the patella. Measurements

were corrected for chondrocyte lacunae. Staining values were corrected for background staining as measured in PG-depleted patellar cartilage in which red stain was no longer visible. PG depletion was induced by intra-articular injection of papain (19). Each experimental group contained at least 8 knee joints, of which 3 semiserial sections were analyzed.

Autoradiographic analysis of ^{35}S-sulfate incorporation was performed as described (18). Radiolabeled sulfate (75 μCi) was injected intraperitoneally 6 hours before dissection of the knee joints. After histologic processing, 6 μm sections were prepared and mounted on gelatin coated slides. These were dipped in K_5 emulsion (Illford Basildon, Essex, UK) and exposed for 3 or 5 weeks. After this period the slides were developed and stained with hematoxylin and eosin.

Determination of patellar cartilage proteoglycan synthesis

Proteoglycan synthesis was measured ex vivo according to the method of van den Berg et al (20). Whole patellae were dissected from the knee joints and pulse-labeled (3 hours at 37 °C) with ^{35}S-sulfate (30 μCi/ml). Subsequently, they were washed, fixed in ethanol and decalcified in formic acid. After decalcification of the patellae, the entire cartilage was stripped off, and a 0.2 mm^2 round section was punched out of the center (central part) remaining the peripheral part (21). The central and peripheral areas of the patellar cartilage were dissolved and ^{35}S-incorporation was counted by liquid scintillation counting. Each experimental group contained at least 6 patellae.

RESULTS

Effect of BMP-2 on articular cartilage PG synthesis in vivo

Intra-articular injection of BMP-2 (2-1000 ng) resulted in stimulation of patellar cartilage PG synthesis which was maximal 2 days after injection. The effect of BMP-2 on PG synthesis was dose dependent with a maximum stimulation of 250% at a dosage $\geq$ 200 ng (data not shown). To investigate whether BMP-2 has differential effects on PG synthesis in the central and peripheral areas of the patella, PG synthesis of both areas was measured separately. BMP-2 stimulated patellar cartilage PG synthesis by both the central and peripheral areas equally (Figure 1A). On day 2 after intra-articular injection of TGF-ß1 (200 ng) no significant effect on articular cartilage PG synthesis was noted (Figure 1A).

Absence of counteraction by BMP-2 of IL-1α-induced PG synthesis inhibition

Since BMP-2 appeared to be a potent stimulator of patellar cartilage PG synthesis we evaluated whether BMP-2 was able to counteract the suppressive effect of IL-1 on cartilage PG synthesis. IL-1α (10 ng) was injected into murine knee joints in the presence or absence of 200 ng BMP-2. Two days after intra-articular injection of Il-1α, total patellar PG synthesis was ~40% lower than in controls. Suppression of PG synthesis by IL-1α alone was higher in the central part (69 $\pm$ 20%) than in the peripheral part (16 $\pm$ 33%) (Figure 1B). Simultaneous injection of 10 ng IL-1α and 200 ng BMP-2 resulted in suppression of PG synthesis to a degree similar to that induced by IL-1α alone (Figure 1B). Coinjection with higher concentrations of BMP-2 (up to 1 μg) had no effect on IL-1α-induced suppression (data not shown). These results demonstrate that although BMP-2 is a potent stimulator of articular cartilage PG synthesis, it is unable to counteract the inhibition of articular cartilage PG synthesis induced by IL-1α. Moreover, these results also indicate that the effects of BMP-2 on cartilage PG synthesis are blocked when chondrocyte metabolism is affected by IL-1α. In parallel with BMP-2, coinjection with TGF-ß1 (200 ng) demonstrated no effect on IL-1α-induced suppression of PG synthesis on day 2 after injection (Figure 1B).

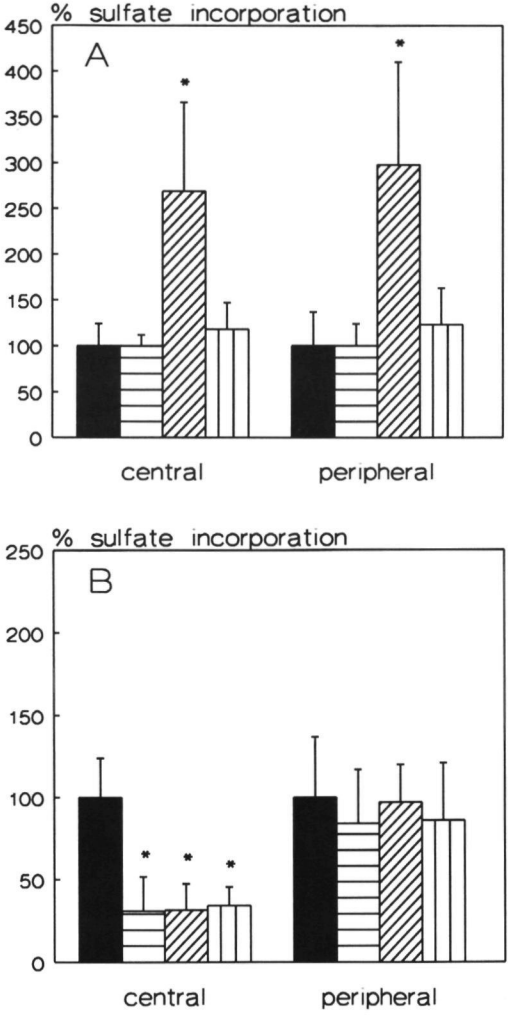

Figure 1 *Patellar cartilage synthesis of proteoglycans in untreated knees (solid bars) and in knees on day 2 after a single intra-articular injection of vehicle (horizontal-striped bars), BMP-2 (200 ng)(hatched bars) or TGF-ß1 (200 ng)(vertical striped bars) without* ***(A)*** *or with* ***(B)*** *co-injection of 10 ng IL-1α. ^{35}S-sulfate incorporation (mean ± SD percentage of incorporation in untreated knees; n=6) was measured using an ex-vivo pulse-labeling of isolated patellae with ^{35}S-sulfate. ^{35}S-sulfate incorporation by central and peripheral areas of untreated knees was comparable (400-700 cpm).*
** P< 0.05 versus vehicle-injected knees, by Student's t-test. (Note: y-axis scales differ in* ***A*** *and* ***B****)*

Effect of long-term BMP-2 exposure on IL-1α-induced inhibition of PG synthesis

In previous studies, we showed that long-term exposure of articular cartilage to TGF-ß1 resulted in stimulation of articular cartilage PG synthesis while short-term exposure had no effect on PG synthesis (22). We sought to determine whether long-term, rather than short-term, exposure of cartilage to BMP-2 could counter IL-1α-induced suppression of PG synthesis. We therefore gave 3 coinjections at alternate days. BMP-2 (200 ng) and TGF-ß1 (200 ng) stimulated articular cartilage PG synthesis in both the central and peripheral part of patellar cartilage (Figure 2A). Coinjections of IL-1α (10 ng) and BMP-2 (200 ng) resulted in a strong suppression (± 60%) PG synthesis in the central part of the patella which was not

significantly different from the synthesis after 3 injections with IL-1α alone (Figure 2B). This indicates that long-term exposure of cartilage to BMP-2 is also not able to counteract the effects of IL-1α.

In contrast to the central region, PG synthesis in the peripheral part in mice injected with IL-1α alone was not different from that of the controls. Knee joints injected with IL-1α (10 ng) and BMP-2 (200 ng) showed a significant stimulation of PG synthesis in the peripheral part of the patella. In contrast to BMP-2, injections of TGF-ß1 (200 ng) in the presence of IL-1α (10 ng) resulted in stimulation of PG synthesis in both the central and peripheral regions of the patellar cartilage (Figure 2B) demonstrating that TGF-ß1 is able to counteract IL-1α-induced suppression of articular cartilage PG synthesis after long-term exposure.

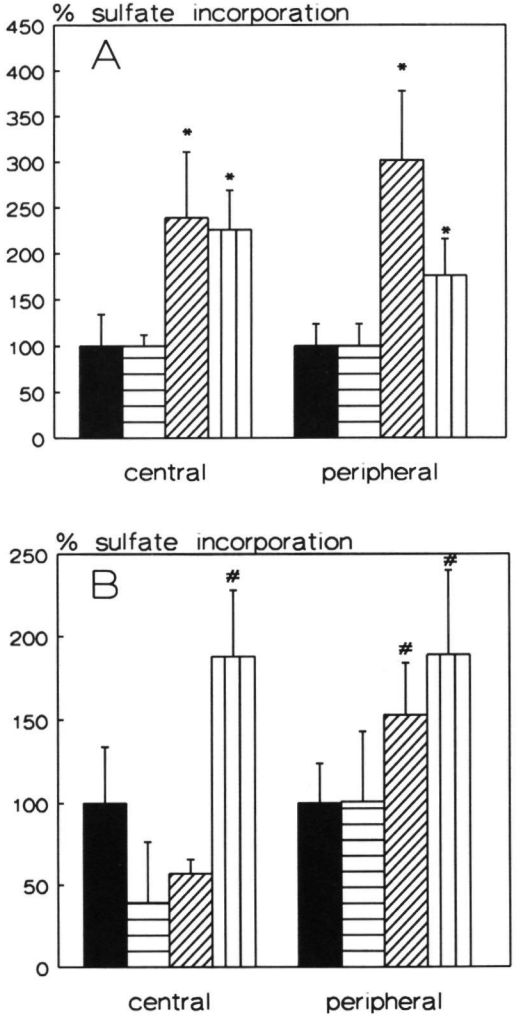

Figure 2 *Patellar cartilage synthesis of proteoglycans in untreated knees (solid bars) and in knees on day 2 after a single intra-articular injection of vehicle (horizontal-striped bars), BMP-2 (200 ng)(hatched bars) or TGF-ß1 (200 ng)(vertical striped bars) without* ***(A)*** *or with* ***(B)*** *co-injection of 10 ng IL-1α. ^{35}S-sulfate incorporation was measured as described in Figure 1, and expressed as the mean ± SD percentage of incorporation of untreated knees (n=6). ^{35}S-sulfate incorporation by central and peripheral areas of untreated knees was comparable (400-700 cpm). * P< 0.05 versus vehicle-injected knees and #=P< 0.05 versus IL-1α-injected knees, by Student's t-test. (Note: y-axis scales differ in* ***A*** *and* ***B****)*

Autoradiographic analysis of local effects of BMP-2 on PG synthesis

To investigate the effects of BMP-2 on chondrocytes from various sites of the joint, PG synthesis was studied by autoradiography on histologic sections. Figure 3 shows autoradiographs of the central regions of the patellar cartilage and the facing femoral cartilage 1 day after 3 injections. BMP-2 (200 ng) stimulated PG synthesis in femoral cartilage to a similar extent as in patellar cartilage and homogeneously throughout the articular cartilage (Figure 3B). Injections of IL-1α (10 ng) resulted in a suppression of PG synthesis in both patellar and femoral cartilage (Figure 3D). Coinjections of IL-1α (10 ng) and BMP-2 (200 ng) suppressed PG synthesis in patellar and femoral cartilage to a similar extent as injections of IL-1α alone (Figure 3E), demonstrating that BMP-2 did not counteract the IL-1α-induced inhibition of PG synthesis in either patellar or the femoral cartilage. In contrast, coinjections of IL-1α (10 ng) and TGF-ß1 (200 ng) resulted in stimulation of PG synthesis in both the patellar and femoral cartilage (Figure 3F) as compared with injections of IL-1α alone (Figure 3D). Similar effects were demonstrated in the femorotibial joint (not shown).

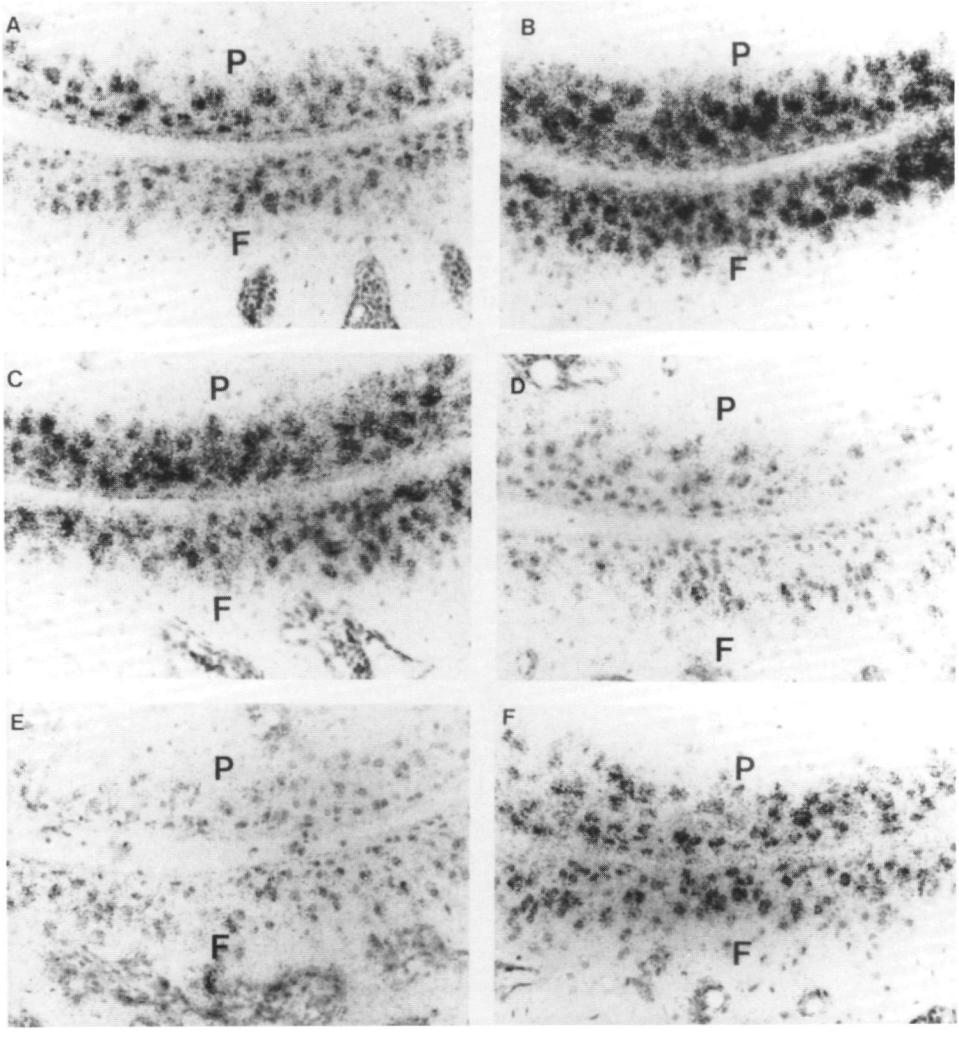

Figure 3 *Autoradiographs showing ^{35}S-sulfate incorporation in the central regions of patellar and femoral cartilage on day 1 after three intra-articular injections of* ***A,*** *vehicle,* ***B,*** *BMP-2 (200 ng),* ***C,*** *TGF-β1 (200 ng)* ***D,*** *IL-1α (10 ng)* ***E,*** *IL-1α (10 ng) + BMP-2 (200 ng) and* ***F,*** *IL-1α (10 ng) + TGF-β1 (200 ng). ^{35}S-sulfate was injected intraperitoneally 6 hours before dissection of the knee joints. After histologic processing autoradiography was performed. (Original magninification X 200).* ***P****=patella;* ***F****=femur*

Effect of BMP-2 on IL-1α-induced PG depletion

Changes in patellar cartilage PG content were measured after triple injections with IL-1α alone or in combination with BMP-2 or TGF-ß1. The PG content of articular cartilage is reflected in the intensity of safranin O staining on histologic sections. As shown in Table 1 significant loss of safranin O staining was noted on day 1 and day 4 after 3 injections of 10 ng Il-1α. Injections with IL-1α (10 ng) in the presence of 200 ng BMP-2 demonstrated that BMP-2 neither affected patellar cartilage PG content on day 1 or day 4. Coinjections of IL-1α (10 ng) and TGF-ß1 (200 ng) resulted in an initial depletion of PGs (day 1 after 3 injections) that was indistinguishable from depletion induced by IL-1α alone. However, 4 days after the last injection patellar cartilage safranin O staining was significantly more intense in joints that had been injected with both IL-1α and TGF ß1 than in those injected with IL-1α alone. These results demonstrate that BMP-2 does not modify IL-1α-induced PG depletion and, in contrast to TGF-ß1, is not able to accelerate the replenishment of PG in the depleted matrix.

Table 1 Safranin O staining on histologic sections of patellar cartilage on day 1 or day 4 after triple intra-articular injections[#]

injected substance	% staining intensity	
	day 1	day 4
vehicle	100 ± 12	100 ± 16
IL-1α (10 ng)	69 ± 12*	76 ± 11*
IL-1α (10 ng)/BMP-2 (200 ng)	71 ± 12*	77 ± 22*
IL-1α (10 ng)/TGF-β 1 (200 ng)	67 ± 14*	107 ± 21†

[#]*Whole knee joints were dissected 1 or 4 days after triple intra-articular injections. Histologic sections were stained with safranin O and quantified using an automated image analyzer. Values were corrected for background staining, as measured in completely depleted patellar cartilage. Each group contains at least 8 knee joints of which 3 histologic sections were analyzed.*

*$P < 0.05$ *versus vehicle-injected knee joints, by Student's t-test.*

†$P < 0.05$ *versus IL-1α-injected knee joints, by Student's t-test.*

Induction of chondrophytes by BMP-2

Histologic sections of knee joints demonstrated that intra-articular injection of BMP-2 (200 ng) resulted in the formation of new chondroid tissues (Figure 4B). These chondrophytes were predominately localized in the patellofemoral area. Chondrophytes were also induced after coinjections of IL-1α (10 ng) and BMP-2 (200 ng)(Figure 4D). This indicates that although IL-1α blocks the effects of BMP-2 on articular cartilage PG synthesis, it does not block the induction of chondrophytes by BMP-2.

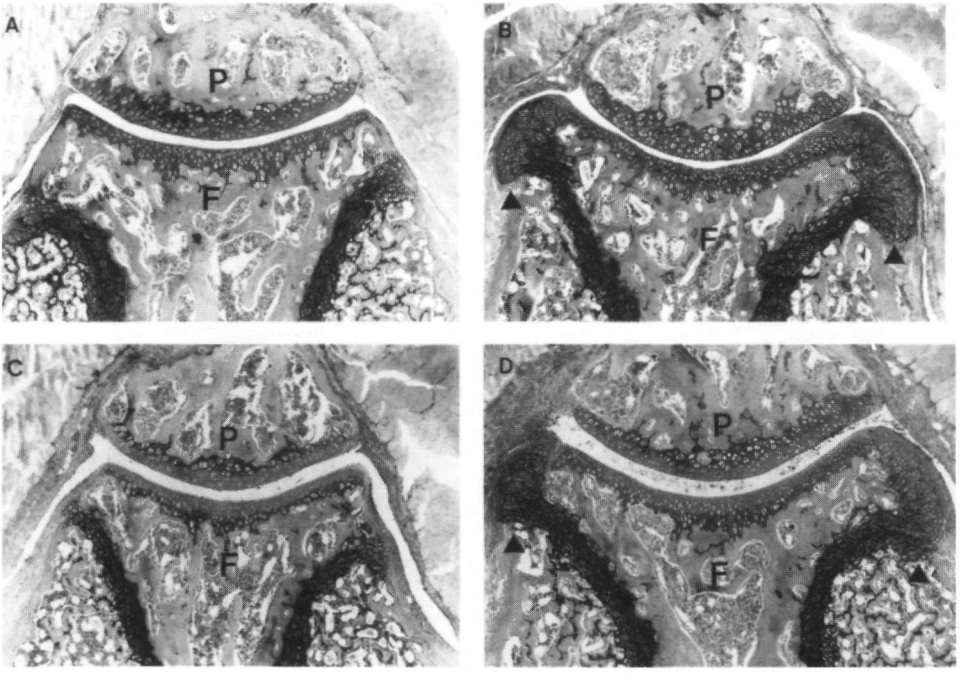

Figure 4 *Histologic features of knee joint sections stained with safranin O showing the induction of chondrophytes by BMP-2 either in the absence or presence of IL-1. Whole knee joints were dissected 1 day after triple intra-articular injections of* ***A,*** *vehicle,* ***B,*** *BMP-2 (200 ng),* ***C,*** *TGF-β1 (200 ng)* ***D,*** *IL-1α (10 ng)* ***E,*** *IL-1α (10 ng) + BMP-2 (200 ng) and* ***F,*** *IL-1α (10 ng) + TGF-β1 (200 ng). (Original magnification X 100).* ***P****=patella;* ***F****=femur;* ***arrows*** *show chondrophytes.*

DISCUSSION

Factors that are able to counteract the deleterious effects of IL-1 on articular cartilage PG synthesis and content or that have the potential to accelerate the replenishment of proteoglycans in depleted cartilage can be expected to be of significant therapeutic value. Because BMP-2 has been demonstrated to be a potent regulator of chondrocyte metabolism and differentiation (12-15), we studied the ability of BMP-2 to stimulate articular cartilage PG synthesis and to counteract the effects of IL-1 on articular cartilage PG synthesis and content in vivo.

This is the first study in which the in vivo effects of BMP-2 on articular cartilage PG synthesis are described. BMP-2 appeared to be a potent stimulator of articular cartilage PG synthesis in vivo. PG synthesis was stimulated homogeneously throughout articular cartilage. In vitro, members of the BMP family like BMP-2, BMP-3, BMP-4 and BMP-7 have been demonstrated to stimulate articular chondrocyte PG synthesis (12,23,24) but until now there were no in vivo data. Also the BMP-2 related factor TGF-ß1 was, as we described before (16,22,25), a potent stimulator of articular cartilage PG synthesis in vivo. Interestingly, the effect of BMP-2 and TGF-ß1 on PG synthesis shows different kinetics. For instance, 1 and 2 days after intra-articular injection of BMP-2 articular cartilage PG synthesis was significantly stimulated whereas injection of TGF-ß1 did not significantly affect PG synthesis before the third day after injection. The difference in kinetics between BMP-2 and TGF-ß1 indicates that in vivo chondrocytes respond immediately to BMP-2 but the TGF-ß1 responses are dependent on a second mediator or on changes in chondrocyte reactivity to TGF-ß1 induced by the injected TGF-ß1 itself.

Although BMP-2 stimulated articular cartilage PG synthesis in vivo, it was unable to counteract the suppression of articular cartilage PG synthesis which was induced by 10 ng of IL-1α. No significant effect of BMP-2 could be demonstrated in cartilage showing IL-1-induced inhibition of PG synthesis. However, it cannot presently be completely excluded that BMP-2 is able to counteract the effects of lower concentrations of IL-1α. Other members of the BMP family have been reported to inhibit IL-1-induced suppression of articular cartilage PG synthesis in vitro. For example, BMP-7 counteracted the effect of IL-1α on chondrocyte PG synthesis in bovine cartilage explants (24). However, no data on the in vivo effects of BMPs on IL-1-induced suppression of articular cartilage PG synthesis have been published until now.

The lack of change in articular chondrocyte PG synthesis by BMP-2 in the presence of IL-1α indicates that the effect of BMP-2 on articular cartilage PG synthesis is blocked when chondrocyte metabolism is affected by IL-1α. The mechanism of IL-1-induced BMP-2 nonresponsiveness is presently unclear at this moment. Downregulation of BMP-2 receptors or blocking of intracellular signaling pathways by IL-1 are 2 possibilities. In contrast to BMP-2, TGF-ß1 was able to counteract IL-1-induced suppression of articular cartilage PG synthesis, indicating that chondrocytes that are affected by IL-1α still have the capability to react to TGF-ß. These results indicate that IL-1α obstructs the BMP-2, but not the TGF-β1 signaling pathway in articular chondrocytes.

Members of the BMP family seem to have, at least in vitro, the potential to inhibit the synthesis of PG degrading enzymes and to inhibit PG breakdown itself. BMP-7 suppressed IL-1-induced up-regulation of collagenase (MMP-1) mRNA and stromelysin (MMP-3) mRNA and counteracted the IL-1-induced inhibition of their natural inhibitor (TIMP) (26). BMP-3 and BMP-4 have been shown to inhibit PG degradation in cartilage explants cultured in vitro (23). We therefore examined the effects of BMP-2 on IL-1α-induced PG depletion in articular cartilage. Safranin O staining of patellar cartilage on histologic sections demonstrated that BMP-2 did not affect IL-1α-induced PG depletion. Although TGF-ß1 appears to be an inhibitor of the catabolic effects of IL-1 on articular cartilage in vitro (27-29), it was unable to inhibit IL-1-induced PG depletion in vivo. The discrepancy between in vitro and in vivo findings could be attributed to mediators produced by synovial cells or by inflammatory cells which are attracted to the joint by co-injections of TGF-ß1 and IL-1 (16).

The ability of BMP-2 to accelerate the replenishment of PGs in IL-1α-depleted cartilage was studied by measuring safranin O staining intensity on day 4 after 3 injections with IL-1α. We demonstrated that BMP-2 did not enhance restoration of PG content in IL-1α-depleted matrix at this point of time. In contrast to BMP-2, TGF-ß1 clearly stimulated repair in the depleted matrix. The different effects of BMP-2 and TGF-ß1 on the replenishment of PGs in the depleted matrix can be explained by the different abilities of BMP-2 and TGF-ß1 to counteract the IL-1-induced suppression of articular cartilage PG synthesis.

Intra-articular injections of BMP-2 resulted in the formation of new chondroid tissue especially in the patellofemoral area. As we demonstrated previously, intra-articular injections of TGF-ß1 also induced chondrophytes (22). Interestingly BMP-2-induced chondrophytes are quite different from TGF-ß1-induced chondrophytes (unpublished observation). The ability of BMP-2 to induce

the formation of new cartilage and bone has been demonstrated before by the rat ectopic bone formation assay (9,30,31). Other members of the BMP family such as BMP-3, BMP-4, BMP-5 and BMP-7 are also able to induce new cartilage and bone in vivo (30-33). Although IL-1α appeared to block the effects of BMP-2 on articular cartilage PG synthesis, the formation of chondrophytes was not inhibited by IL-1α which demonstrates that IL-1 does not block all BMP-2-mediated responses in the joint.

In summary, this study demonstrates that BMP-2 is a potent stimulator of articular cartilage PG synthesis. However, when chondrocyte metabolism is affected by IL-1α, the stimulatory effect of BMP-2 on PG synthesis is completely blocked. Because IL-1 is present in arthritic joints (34-37) our results indicate that BMP-2 alone cannot be used to stimulate cartilage repair during arthritis. Although BMP-2 appears to be unable to stimulate cartilage repair when IL-1 is present, BMP-2 might stimulate cartilage repair in the presence of IL-1 inhibitors such as IL-1 receptor antagonist (IRAP). In addition, BMP-2 could have the potential to stimulate cartilage repair in pathologic conditions, such as cartilage trauma, in which it is unlikely that IL-1 is involved. However, formation of chondrophytes might limit the therapeutic applications of BMP-2.

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CHAPTER 6

STIMULATION OF ARTICULAR CARTILAGE REPAIR IN ESTABLISHED ARTHRITIS BY LOCAL ADMINISTRATION OF TRANSFORMING GROWTH FACTOR β INTO MURINE KNEE JOINTS

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SUMMARY

A severe consequence of rheumatoid arthritis is depletion of proteoglycans (PGs) from articular cartilage leading to functional impairment of this tissue. We investigated whether local administration of anabolic factors (TGF-β1, TGF-β2 and BMP-2) into joints could stimulate cartilage repair during arthritis. An unilateral arthritis was induced in mice by intra-articular injection of zymosan. Starting on day 4 after the induction of arthritis, three injections with 200 ng transforming growth factor β (TGF-β1) were given (days 4, 6 and 8). On day 11 articular cartilage PG synthesis was measured by ^{35}S-sulfate incorporation and histologic knee joint sections were prepared which were used to analyze cartilage PG content by quantification of safranin O staining. Additionally, histologic sections were used to analyze inflammation and chondrophyte-formation. Local administration of TGF-β1 did not modify inflammation but clearly stimulated PG synthesis and restored PG content of depleted cartilage. TGF-β2 appeared to be as potent as TGF-β1 in the stimulation of cartilage repair. Both TGF-β isoforms stimulated in addition to cartilage repair the formation of chondrophytes in this rodent model. In contrast to TGF-β, 3 intra-articular injections with 200 ng bone morphogenetic protein 2 (BMP-2) did not stimulate the repair process. In summary, this study demonstrates for the first time that local administration of TGF-β into arthritic joints stimulates the replenishment of PGs in depleted cartilage.

INTRODUCTION

Rheumatoid arthritis is a disease which is characterized by inflammation of the joints. A severe consequence of the inflammation is degradation of articular cartilage of which depletion of proteoglycans (PGs) is an early event. PGs are highly negatively charged proteins which are responsible for the mechanical properties of articular cartilage. Depletion of proteoglycans is a result of a suppressed PG synthesis and an elevated PG degradation. Suppression of PG synthesis appears to be mediated by cytokines of which interleukin-1 (IL-1) has been demonstrated to be a key mediator (1-3). Degradation of PG seems to be the result of cytokine-induced upregulation and activation of matrix-degrading enzymes like metalloproteinases and the putative PG degrading enzyme aggrecanase (4-8).

Articular cartilage has a limited capacity for self renewal. Anabolic factors which are able to increase PG synthesis and to decrease PG degradation might stimulate cartilage repair in arthritic joints. In this respect transforming growth factor β (TGF-β) seems to be promising. TGF-β1 has been demonstrated to stimulate PG synthesis of articular chondrocytes in vitro and in vivo (9-11). In addition, TGF-β1 is able to counteract IL-1-induced suppression of cartilage PG synthesis (12,13) and has the capacity to reduce IL-1-induced PG depletion in vitro (12,14,15). The latter effect appears to be the result of TGF-β-induced down regulation of the synthesis of proteolytic enzymes and upregulation of the production of enzyme inhibitors (14,16-18).

The purpose of this study was to investigate whether local administration of exogenous TGF-β1 stimulates articular cartilage repair in arthritic joints in which significant PG degradation has already occurred. In addition, the effects of TGF-β1 were compared with the effects of two closely related growth factors: TGF-β2 and bone morphogenetic protein-2 (BMP-2). TGF-β2 is a TGF-β-isoform that has a 71% (amino acid) homology with TGF-β1 (19). Although TGF-β1 and TGF-β2 are quite homologous, their affinities to membrane TGF-β receptors are different, possibly giving rise to different modulating effects on PG depletion in arthritic joints (20-22). BMP-2 was used since this member of the TGF-β superfamily has been demonstrated to be a very potent stimulator of articular cartilage PG synthesis in vitro as well as in vivo (23,24).

MATERIALS AND METHODS

Animals

Male C57Bl/6 mice between 8 and 12 weeks of age were used. They were fed a standard diet and tap water *ad libitum*.

Growth factors

Recombinant human TGF-ß1 (0.1 mg/ml in 20 mM NaOAc pH 5.0) and recombinant human TGF-β2 (1mg/ml in 5% Acetic acid) were supplied by Genentech Inc (South San Francisco, USA) and Novartis Farma Inc (Basle, Switzerland) respectively. Recombinant human BMP-2 (2.27 mg/ml in 0.5M Arginine, 10 mM Histidine pH 6.5) was kindly provided by Elisabeth Morris (Genetics Institute Inc,Cambridge, USA). To prevent loss of protein due to adherence to plastic only siliconized tubes and tips were used.

Induction of zymosan-induced arthritis

To induce an unilateral arthritis in the right mouse knee joints zymosan was intra-articularly injected. A homogeneous suspension of zymosan A (*Saccharomyces cerevisiae*) was obtained after boiling twice and sonic emulsification of a suspension of 30 mg zymosan A in 1 ml endotoxin-free saline. Monoarticular arthritis was induced by injection of 6 μl of the suspension (180 μg zymosan A) into the right knee joint.

Injections of growth factors into arthritic knee joints

Local administration of TGF-β1, TGF-β2 or BMP-2 into arthritic knee joints was carried out by intra-articular injections of 6 μl growth factor (20 and 200 ng) diluted in physiological saline + 0.1 % bovine serum albumin. Dose-response studies performed earlier in our laboratory demonstrated that intra-articular injection of 200 ng TGF-β1, TGF-β2 or BMP-2 is optimal for stimulation of patellar cartilage PG synthesis in mice (25).

Histology

Whole knee joints were dissected and fixed for 7 days in phosphate-buffered formalin. The fixed knee joints were decalcified (5% formic acid) and dehydrated by an automated tissue processing apparatus (VIP, Miles Scientific, Naperville, IL). After embedding in paraffin wax semiserial frontal knee sections (6 μm) were prepared and mounted on gelatin-coated slides. Paraffin was removed by xylol and ethanol whereafter sections were stained with safranin O and fast green (26). Safranin O staining, a semi-quantitative marker of PG depletion, was measured using an automated image analysis system (VIDAS, Kontron Electronics, Munich, Germany) (27). Fast green staining was neutralized by use of a green filter. Optical density was examined in the non-calcified cartilage of the patella. Measurements were corrected for chondrocyte lacunae. Staining values were corrected for background staining, as measured in PG-depleted patellar cartilage in which red stain was no longer visible.
The histologic sections were also scored by blinded observers for synovitis and the presence of chondrophytes. For both parameters a scale from 0 (no synovitis/chondrophytes) to 3 (severe synovitis/chondrophyte-formation) was used. Chondrophytes were scored on three different locations: adjacent to the patella, nearby the cartilage border of the femur (in the patellofemoral area) and nearby the insertions of collateral ligaments on the femur.

Determination of patellar cartilage proteoglycan synthesis

Proteoglycan synthesis was measured ex vivo according to the method of van den Berg et al (28). Whole patellae were dissected from the knee joints and pulse-labeled (3 hours, 37 °C) with ^{35}S-sulfate (30 μCi/ml). Subsequently they were washed, fixed in ethanol and decalcified in formic acid. After separation of the cartilage layer from the underlying bone the cartilage was dissolved in Lumasolve (Hicol, Oud-Beijerland, the Netherlands) and ^{35}S-incorporation was counted by liquid scintillation counting. Each experimental group contained at least 6 patellae.

RESULTS

Intra-articular injection of zymosan inhibits cartilage PG synthesis and induces PG depletion

Intra-articular injection of zymosan into murine knee joints resulted in arthritis characterized by pronounced influx of inflammatory cells in the synovium and joint cavity. Suppression of PG synthesis (~ 45%) and loss of safranin O staining (~ 40%) were present on day 4 after induction of arthritis. PG synthesis inhibition at day 11 was variable between multiple experiments (range 0-70%) reflecting variation in the moment of waning of arthritis and normalization of chondrocyte function. However, significant PG depletion was always observed at this point of time. Injections of physiological saline plus 0.1% BSA on days 4, 6 and 8 did not affect PG synthesis or safranin O staining as measured on day 11 (data not shown).

Local administration of TGF-ß1 into arthritic knee joints stimulates articular cartilage PG synthesis

We studied whether local adminstration of exogenous TGF-ß1 into arthritic joints results in stimulation of cartilage PG synthesis. Three injections with 20 or 200 ng TGF-ß1 were given on days 4, 6 and 8 respectively. As can be seen in figure 1, on day 11 after arthritis induction patellar cartilage PG synthesis in joints which received 3 injections with TGF-ß1 was clearly stimulated when compared to saline-injected joints. PG synthesis was even significantly higher in TGF ß-injected arthritic joints as compared to normal, untreated joints. A dose of 200 ng TGF ß1 appeared to be more potent in stimulating PG synthesis than 20 ng. These results demonstrate that suppression of PG synthesis in arthritic joints can be abolished by local administration of TGF-ß1.

Enhanced PG replenishment in depleted cartilage by local administration of TGF-ß1

It was investigated if TGF ß1, in addition to stimulation of PG synthesis, could stimulate the replenishment of proteoglycans in depleted cartilage. Proteoglycan content was studied by analysis of safranin O staining of patellar cartilage on day 11 after induction of arthritis. Safranin O staining of patellar cartilage from arthritic, saline-injected joints (controls), was significantly reduced compared to safranin O staining of patellar cartilage from normal, untreated joints (Figure 2). However, arthritic joints which

received 3 injections with 200 ng TGF-ß1 demonstrated no significant loss of staining, compared to normal knees. Injections with 20 ng TGF-ß1 showed also increased proteoglycan content but the effect was less pronounced with 20 as compared with 200 ng TGF-β1 (Figure 2). Since pronounced loss (~40%) of safranin O staining was already present on day 4 these data show that injections of TGF-ß1 in arthritic joints stimulate the replenishment of PGs in depleted cartilage (Figure 3).

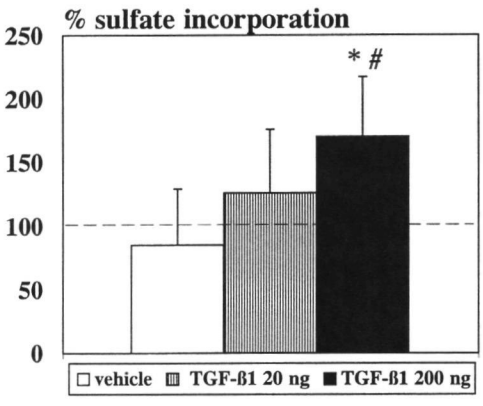

Figure 1 *Patellar cartilage synthesis of proteoglycans in arthritic joints after three intra-articular injections with vehicle, 20 ng TGF-β1 or 200 ng TGF-β1. Monoarticular arthritis was induced by injection of 180 μg zymosan into the right knee joint. Intra-articular injections were given on days 4, 6 and 8. On day 11, ^{35}S-sulfate incorporation (mean ± SD percentage of incorporation in untreated, left knees; n=18) was measured after an ex vivo pulse-labeling of isolated patellae. * = P< 0.05 versus vehicle-injected knees, by Student's t-test. # = P<0.05 versus untreated, left knees, by Students's t-test.*

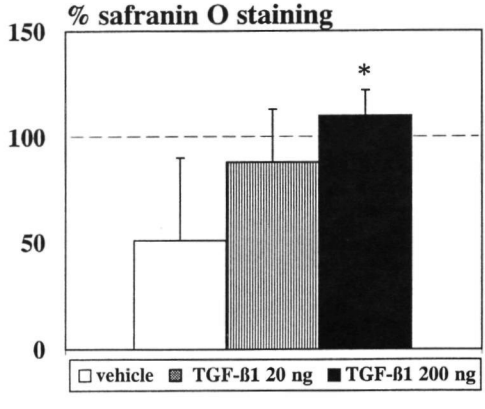

Figure 2 *PG content as measured by safranin O staining of articular cartilage from arthritic joints after three intra-articular injections with vehicle, 20 ng or 200 ng TGF-β1. Monoarticular arthritis was induced by injection of 180 μg zymosan into the right knee joint. Intra-articular injections were given on days 4, 6 and 8. On day 11, whole knee joints were dissected and histologic sections were prepared which were stained with safranin O. Staining was quantified using an automated image analyzer. Values (mean ± SD percentage of staining in untreated, left knees) were corrected for background staining, as measured in completely depleted patellar cartilage. * = P< 0.05 versus vehicle-injected knees, by Student's t-test (n=18).*

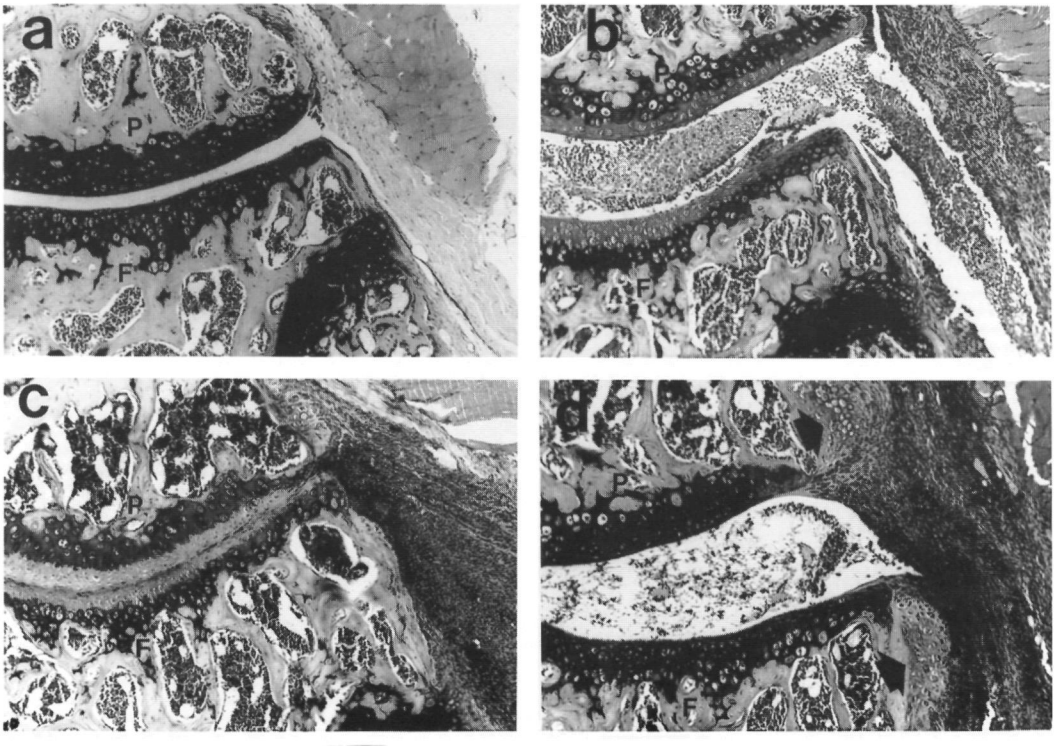

Figure 3 *Histology of arthritic knee joints showing restoration of articular cartilage safranin O staining and stimulaton of chondrophyte-formation after intra-articular injections with 200 ng TGF-β1. Monoarticular arthritis was induced by injection of 180 μg zymosan into the right knee joint. Intra-articular injections with vehicle or 200 ng TGF-β1 were given on days 4, 6 and 8. On day 4 and 11, whole knee joints were dissected. A, normal, untreated knee joint; B, arthritic knee joint before intra-articular injections (day 4). C, arthritic knee joint after 3 injections with vehicle (day 11). D, arthritic knee joint after 3 injections with 200 ng TGF-β1 (day 11). (Original magnification X100). P = patella; F= femur; arrows indicate chondrophytes.*

Stimulation of chondrophyte-formation by intra-articular injections of TGF-β1

To study if administration of TGF-β modulates the inflammation and the formation of chondrophytes histologic sections were analyzed. As shown in table 1 no significant differences in synovitis were observed between arthritic joints which received injections with physiological saline and joints which were injected with TGF-ß1.

The zymosan-arthritis model we used shows moderate formation of chondrophytes in the knee joint on day 11 as can be seen in table 1. Analysis of the histologic sections clearly demonstrated that the development of these chondrophytes at different locations in the arthritic joints was stimulated by the injections of 200 ng TGF-β1 (Table 1, Figure 3). In contrast, injections with 20 ng TGF-ß1 did not significantly enhance chondrophyte-formation on day 11.

Table 1 Histologic analysis of the effect of 3 intra-articular injections of 20 ng or 200 ng TGF-β1 into arthritic knee joints on synovitis andchondrophyte-formation

		Chondrophytes		
treatment	synovitis	A	B	C
saline	2.0 ± 0.8	0.5 ± 0.4	0.1 ± 0.1	0.6 ± 0.3
20 ng TGF-β1	1.9 ± 0.9	0.7 ± 0.4	0.1 ± 0.2	0.9 ± 0.5
200 ng TGF-β1	2.4 ± 0.6	1.5 ± 0.5*	0.8 ± 0.6*	1.5 ± 0.4*

*Monoarticular arthritis was induced by injection of 180 μg zymosan into the right knee joint. Intra-articular injections were given on days 4, 6 and 8. Whole knee joints were dissected on day 11. Histologic sections were scored by blinded observers for synovitis and the presence of chondrophytes. A scale from 0-3 was used. Chondrophytes were scored on three different locations: adjacent to the patella (A), nearby the cartilage border of the femur (in the patellofemoral area) (B) and at the insertions of collateral ligaments on the femur (C). * = P< 0.05 versus vehicle-injected knees, by Wilcoxon's rank sum tests (n=16).*

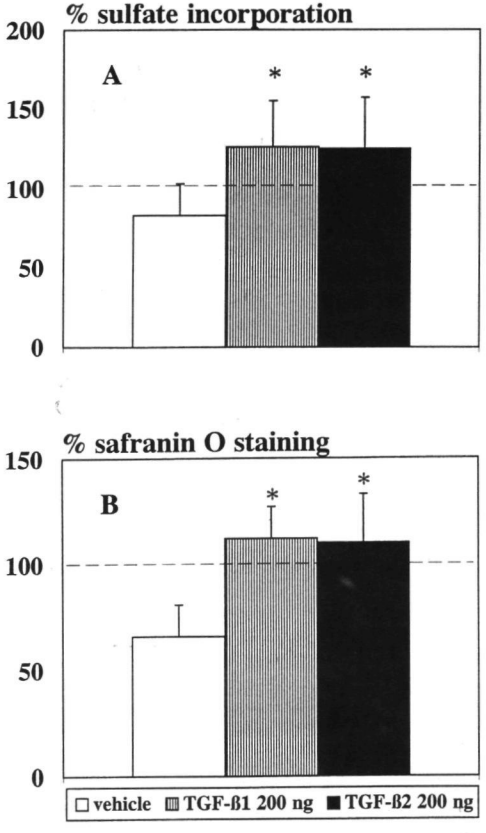

*Figure 4 Synthesis of proteoglycans (A) and staining with safranin O (B) of patellar articular cartilage from arthritic joints receiving three intra-articular injections with vehicle, 200 ng TGF-β1 or 200 ng TGF-β2. Monoarticular arthritis was induced by injection of 180 μg zymosan into the right knee joint. Intra-articular injections were given on days 4, 6 and 8. On day 11, patellae and whole knee joints were dissected for ^{35}S-sulfate incorporation and preparation of histologic sections. Values are expressed as mean ± SD percentage of values of untreated, left knees; n=8). * = P< 0.05 versus vehicle-injected knees, by Student's t-test.*

TGF-β1 and TGF-β2 are equally potent in the stimulation of cartilage repair

To investigate whether the ability of TGF-ß to stimulate cartilage repair in arthritic joints is isoform-specific, the effects of TGF-ß1 and TGF-ß2 on PG synthesis and content were compared. As shown in figure 4 both TGF-ß1 and TGF-ß2 stimulated cartilage PG synthesis in arthritic joints. Significant differences between TGF-β1 and TGF-β2 could not be demonstrated. No depletion of PG was observed in joints which were injected with TGF-β1 or TGF-β2, while PG depletion was clearly demonstrated in the physiological saline injected joints (Figure 4). This indicates that both TGF-β isoforms stimulate replenishment of PGs in depleted cartilage. Stimulation of chondrophyte-formation, which was observed after injections of TGF-β1, was also demonstrated after injections with TGF-β2 (Table 2).

Table 2 Histologic analysis of the effect of 3 intra-articular injections of 200 ng TGF-β1 or 200 ng TGF-β2 into arthritic knee joints on the formation of chondrophytes

	Chondrophytes		
treatment	A	B	C
saline	0.4 ± 0.4	0.1 ± 0.1	0.8 ± 0.2
200 ng TGF-β1	1.7 ± 0.4*	0.6 ± 0.4*	1.7 ± 0.3*
200 ng TGF-β2	1.9 ± 0.6*	1.0 ± 0.5*	1.2 ± 0.3*

*Monoarticular arthritis was induced by injection of 180 μg zymosan A into the right knee joint. Intra-articular injections were given on days 4, 6 and 8. Whole knee joints were dissected on day 11. Histologic sections were scored by blinded observers for the presence of chondrophytes. A scale from 0-3 was used. Chondrophytes were scored on three different locations: adjacent to the patella (A), nearby the cartilage border of the femur (in the patellofemoral area) (B) and at the insertions of collateral ligaments on the femur (C). * = $P < 0.05$ versus vehicle-injected knees, by Wilcoxon's rank sum tests ($n=8$).*

TGF-β is more potent than BMP-2 in the stimulation of cartilage repair in arthritic joints

We have previously demonstrated that BMP-2 is a very potent stimulator of articular cartilage PG synthesis in vivo, therefore the ability of BMP-2 to stimulate cartilage repair in arthritic joints was studied. BMP-2 was, in contrast to TGF-β1, unable to stimulate PG synthesis in arthritic joints (Figure 5A). Analysis of safranin O staining on histologic sections showed similar loss of staining in vehicle-injected and BMP-2-injected joints. In contrast, safranin O staining of TGF-β-injected joints was not different from normal, untreated joints (Figure 5B). These results show that local administration of BMP-2 into arthritic joints has, in contrast to local administration of TGF-β, no effects on cartilage repair.

A single injection of 200 ng TGF-β2 into arthritic joints does not affect cartilage PG synthesis and content

The observation of enhanced induction of chondrophytes by injection of TGF-β into arthritic joints might limit potential therapeutic application of local TGF-β administration. In an attempt to stimulate cartilage repair without the formation of chondrophytes we studied the effects of an alternative protocol of TGF-β administration. Studies of our group showed that a single injection of 200 ng TGF-ß stimulated cartilage PG synthesis in normal joints for several weeks without the induction of chondrophytes (25). Therefore a protocol in which a single injection of

200 ng TGF-ß2 was given at day 3 or day 4 after the induction of arthritis was tested. However, administration of 200 ng TGF-ß2 in arthritic joints using this protocol neither stimulated PG synthesis nor increased PG content (data not shown).

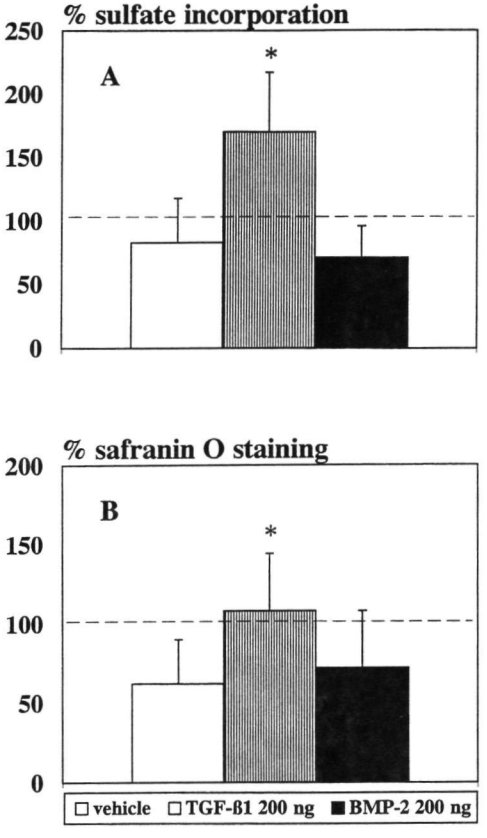

Figure 5 *Synthesis of proteoglycans* ***(A)*** *and staining with safranin O* ***(B)*** *of patellar articular cartilage from arthritic joints receiving three intra-articular injections with vehicle, 200 ng TGF-β1 or 200 ng BMP-2. Monoarticular arthritis was induced by injection of 180 μg zymosan into the right knee joint. Intra-articular injections were given on days 4, 6 and 8. On day 11, patellae and whole knee joints were dissected for ^{35}S-sulfate incorporation and preparation of histologic sections. Values are expressed as mean ± SD percentage of values of untreated, left knees; n=8). * = P< 0.05 versus vehicle-injected knees, by Student's t-test.*

DISCUSSION

We investigated whether local administration of exogenous TGF-ß into murine arthritic knee joints stimulates repair of PG-depleted articular cartilage. We demonstrated in this study that local administration of exogenous TGF-ß into the joints stimulates cartilage PG synthesis and enhances the replenishment of PG in the depleted matrix. No data about the presence of active TGF-ß in synovial fluid of murine arthritic knee joints are available but high concentrations of latent and active TGF-β have been demonstrated in synovial fluids of human rheumatoid arthritis patients (29-32). However, although active TGF-ß might be present in

murine arthritic joints, local administration of exogenous TGF-ß into these joints results in profound effects on cartilage PG synthesis and content.
In the zymosan-induced arthritis model used, it appeared that three injections of TGF-ß1 abolished the suppression of cartilage PG synthesis and even stimulated PG synthesis to a supranormal level. In this model of experimental arthritis IL-1 has been shown to be a key mediator in the suppression of PG synthesis (3). The ability of TGF-β to abolish suppression of cartilage PG synthesis during zymosan-induced arthritis is in line with the ability of TGF-β to counteract the effects of IL-1 on PG synthesis which has been demonstrated in vitro and in vivo by our group (12,13). The mechanism of this action is unclear but the abolishment of IL-1-effects might be the result of TGF-β-induced down-regulation of IL-1 receptors (33,34) or suppression of nitric oxide (NO) synthesis (35,36), a factor involved in IL-1-induced suppression of PG synthesis (37,38).

Zymosan-induced arthritis resulted in a significant depletion of PG from articular cartilage at day 4. No significant PG depletion was demonstrated on day 11 in joints which received three intra-articular injections of 200 ng TGF-β1 starting at day 4, although a severe and similar inflammation was present in the control joints and TGF ß-injected joints. Because PG depletion was already demonstrated at day 4 after arthritis induction it can be concluded that injections of TGF-β1 stimulated replenishment of PG. Stimulation of PG replenishment in depleted cartilage by TGF-β has been described before in vitro and in vivo (13,39,40) but this is the first report of replenishment of PG in depleted articular cartilage during persistent arthritis. It is likely that stimulation of PG synthesis by TGF-β plays an important role in the enhanced replenishment of PG in depleted cartilage but also the concominant stimulation of the synthesis of hyaluronic acid by TGF-ß (10) might contribute to the repair process. Moreover, TGF-β might enhance the retention of newly synthesized matrix molecules by increasing the expression of integrins on chondrocytes (41,42).

It has been described that TGF-β can differentially regulate the expression of aggrecan, biglycan and decorin. TGF-β enhanced biglycan synthesis and decreased decorin synthesis in chondrocytes (43), fibroblasts (44,45) and osteosarcoma cells (45). In addition, in cultures of meniscal cells TGF-β stimulated the expression of biglycan but did not affect the expression of decorin (46). However, after intra-articular injection of TGF-β into normal murine knee joints a consistent upregulation of aggrecan mRNA expression but not of biglycan and decorin mRNA expression could be demonstrated (25). In addition to differential regulation of PG expression by TGF-β, it has been shown that TGF-β affects the size and

degree of sulfation of PG glycosaminoglycan chains chains (10,44,47). Because of the differential regulation of PG expression and the effects of TGF-β on glycosaminoglycan chains it would be of great interest to characterize the newly synthesized PGs in the TGF-β-injected arthritic knee joints. However, due to the small size of mice and consequently limited amount of cartilage, it is almost impossible to characterize the nature of the newly synthesized PGs in vivo.

In addition to the effects on matrix synthesis, local administration of TGF-ß might also enhance cartilage repair by suppression of PG degradation. TGF-β1 has been demonstrated to inhibit IL-1-induced matrix degradation (12,15) probably by downregulating the synthesis of matrix degrading enzymes (14,16,17) and upregulation of tissue inhibitor of metalloproteinases (TIMP) synthesis (16-18). TGF-ß-mediated upregulation of plasminogen activator inhibitor-1 (PAI-1) (48,49) might also play a role since plasmin has been suggested to be involved in cartilage degradation by direct cleavage of matrix molecules or by the activation of latent metalloproteases (50-52).

Stimulation of cartilage repair in arthritic joints after local adminstration of TGF-ß appears mainly to be a direct effect of TGF-ß on chondrocyte metabolism since this treatment did not reduce inflammation. However, suppression of inflammatory cell activity by TGF-ß might also play a role. TGF-β has been demonstrated to suppress the release of reactive oxygen, nitrogen metabolites and cytokines by activated macrophages (53-55) and has been described to down-regulate the expression of receptors for tumour necrosis factor-α (TNF-α) and IL-1 on several types of cells (56,57).

To study whether the TGF-β-induced stimulation of cartilage repair is isoform-specific the effects of TGF-β1 and TGF-β2 were compared. Both isoforms have been described to be equally potent in the suppression of IL-1-induced expression of metalloproteinases by articular chondrocytes in vitro (17,33) but seem to have different abilities to inhibit H_2O_2 release by activated macrophages (53) and to stimulate chondrogenesis in vivo (58). In our study we demonstrated that TGF-β2 stimulated articular cartilage PG synthesis, replenishment of PG in depleted cartilage, and chondrophyte-formation to a similar extent as TGF-β1. However, additional dose-response studies are required for more detailed comparison of the relative potency of the two isoforms.

BMP-2 is a very potent stimulator of articular cartilage PG synthesis in vitro (23) and in vivo (24,25). In addition, BMP-2 has been shown to accelerate healing of full-thickness cartilage defects in vivo (59). Therefore it was studied if local administration of BMP-2 could accelerate cartilage repair in arthritic joints. Intra-

articular injections of BMP-2 did not stimulate articular cartilage PG synthesis nor stimulated the replenishment of PG in depleted matrix. The disability of BMP-2 to stimulate cartilage PG synthesis in arthritic joints might be attributed to the presence of IL-1 in these joints. Recently we demonstrated in an in vivo study that chondrocytes were totally unresponsive to the stimulating effects of BMP-2 shortly after exposure to IL-1 (24). Local administration of TGF-β into arthritic joints was beneficial for cartilage without significant effects on inflammation. However, there are also studies suggesting a pathogenic role of TGF-ß during arthritis. Intra-articular injection of TGF-β into normal rat or murine knee joints has been described to induce influx of inflammatory cells and to induce synovial hyperplasia (13,60-62). In addition, intra-articular injection of TGF-β in mice immunized with collagen type II stimulated the onset of arthritis (63) and neutralization of TGF-β just before induction of streptococcal cell wall (SCW)-induced arthritis in rats reduced acute and chronic arthritis (64). This indicates that endogenous TGF-β could be proinflammatory in the early phase of arthritis but might protect against cartilage degradation and is able to stimulate repair in established arthritis.

Although this study demonstrates that local administration of TGF-ß into arthritic knee joints stimulates cartilage repair, therapeutic application of this treatment might be limited by the formation of chondrophytes. The ability of TGF-ß to induce chondrophytes in murine knee joints is in line with previous reports from our group (11,13). To reduce the formation of chondrophytes a lower TGF ß dose was used. No significant stimulation of chondrophyte-formation was observed after three injections of 20 ng TGF-β but this protocol was also less effective in stimulation of cartilage repair. In another protocol a single instead of three intra-articular injections of 200 ng TGF-β were given. However, also this treatment failed to stimulate cartilage repair. The formation of chondrophytes and osteophytes is observed in many models of experimental arthritis in rodents but is hardly seen in human patients with rheumatoid arthritis. This might indicate that periosteum is less responsive to chondrophyte-inducing factors in human arthritic joints as compared with murine periosteum and that TGF-ß-induced formation of chondrophytes and osteophytes might be a minor problem in humans. This warrants testing in non-rodent animal models before clinical application in humans is an option.

In summary, this study demonstrates that local administration of TGF-ß1 into arthritic joints stimulates the replenishment of PG in depleted cartilage. The ability of TGF-ß1 to stimulate cartilage repair in established arthritis was shared with TGF-ß2 but not with BMP-2.

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CHAPTER 7

OSTEOARTHRITIS-LIKE CHANGES IN THE MURINE KNEE JOINT RESULTING FROM INTRA-ARTICULAR TRANSFORMING GROWTH FACTOR BETA INJECTIONS

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ABSTRACT

In earlier studies we have shown that intra-articular injections of transforming growth factor beta (TGF-ß) stimulated murine articular cartilage proteoglycan synthesis and increased proteoglycan content. Moreover, in these short-term experiments we also found formation of new cartilage-like tissues at the margins of articular cartilage. In the present study we investigated the long-term effects of intra-articular TGF-β injections. TGF-ß was injected once or repeatedly, whereafter articular cartilage proteoglycan synthesis and content were studied over a 2 months period. In addition, histologic sections of total knee joints were analyzed. Besides longterm elevation of proteoglycan synthesis and content in articular cartilage, no changes could be demonstrated in knee joints after a single injection of TGF-ß. In contrast, one month after triple TGF-ß injections lesions with severe proteoglycan depletion were found in deeper layers of the posterior part of the lateral tibia while the superficial layer of tibial cartilage still had an increased proteoglycan content. Additionally, multiple TGF-ß injections induced newly formed chondroid tissues along the margins of articular cartilage which were transformed into osteophytes via calcification of this tissue. Besides the induction of these osteophytes, TGF ß-induced chondroid tissue appeared in medial collateral ligaments. All these TGF-ß-induced changes resemble features of experimental and spontaneous osteoarthritis in mice, suggesting a role for TGF-ß in the pathogenesis of osteoarthritis.

INTRODUCTION

In search for an anabolic agent for cartilage repair and regeneration, transforming growth factor beta (TGF-ß) has been considered a suitable candidate. TGF-ß is a multipotent regulator of cell growth and differentiation, and of extracellular matrix production. Three different isoforms have been found in mammalian species: TGF-ß1, -ß2, and -ß3. The mature TGF-beta's are all 25 kDa homodimers, with each monomer consisting of 112 amino acids containing 9 cysteine residues. TGF-ß is normally secreted as an inactive high molecular weight complex which has to be dissociated before activation. High levels of active TGF-ß have been found in synovial fluids of rheumatoid arthritis and osteoarthritis (OA) patients (1,2), indicating that this factor is produced and activated during joint pathology.

In earlier studies (3,4) we have shown that intra-articular injection of TGF-ß1 or TGF-ß2 stimulated articular cartilage proteoglycan synthesis during one week. Moreover, TGF-β counteracted suppression of proteoglycan synthesis by interleukin-1 (IL-1), and accelerated proteoglycan replenishment of depleted articular cartilage, suggesting that TGF-ß protects articular cartilage during pathology. However, administration of TGF-ß into murine knee joints also induced development of cartilage-like structures in periosteum, at sites where osteophytes develop in murine arthritis and osteoarthritis models, suggesting a role for TGF-ß in osteophyte formation during joint pathology. Because both osteophyte formation and enhanced articular cartilage proteoglycan synthesis are found not only after TGF-ß injections, but also in early experimental osteoarthritis (5,6), one might consider excessive TGF-ß levels as a causative factor in OA. In analogy, a similar role of TGF-ß has been emphasized in tissue fibrosis in kidney and liver diseases and excessive scar formation in skin healing, and has been termed the dark side of tissue repair (7,8). In the present study we investigated changes in cartilage and other joint structures, over a two-months period after single and repeated injections of TGF-ß into the murine knee joint.

MATERIALS AND METHODS

Animals

Male C57Bl/10 mice aged 12 weeks or 18 months were used. They were fed a standard diet and tap water ad libitum.

Intra-articular injections

After anaesthetizing the mice with ether, 200 ng recombinant human TGF-ß1 (Genentech Inc., San Francisco, CA, USA.) dissolved in 6 μl of physiological saline (0.9% NaCl) + 0.1% ultrapure bovine serum albumin (Sigma, St Louis, MO, USA.) was injected into the joint cavity of the right knee once or three times at alternate days. To study whether the effects of triple TGF-β injections can be augmented, additional injections of TGF-β were given in some experiments. Experimental osteoarthritis was induced by intra-articular injection of 10 units of highly purified collagenase (type VII, Clostridium histolyticum, Sigma, St Louis, MO, U.S.A) dissolved in 6 μl physiological saline (9,10).

Determination of patellar cartilage proteoglycan synthesis

Proteoglycan synthesis was measured ex vivo. Whole patellae, with a standard amount of surrounding tissue, were dissected from the knee joints. Patellae were then pulse-labeled (2 hours at 37°C) with ^{35}S-sulfate. Subsequently, they were washed, fixed, decalcified, punched out of surrounding tissue, and dissolved as described before (11). The ^{35}S-content of each patella, which is a reliable measure of patellar cartilage proteoglycan synthesis (12), was measured by liquid scintillation counting.

Determination of patellar cartilage proteoglycan content

Articular cartilage proteoglycan content is reflected by safranine O staining intensity in histologic sections. This was measured, as described before (13), using an automated image analysis system (VIDAS, Kontron Elektronik GMBH). Microscopic images were recorded by a CDD video camera (Sony) and processed by a personal computer. Optical density was measured by integral measurement in a 20 μm layer, along the cartilage surface. The total zone of non-calcified cartilage is approximately 30-40 μm in width. Fast green staining was neutralized by use of a green filter. Measurements were corrected for lacunae. Proteoglycan content in articular cartilage of TGF-ß-injected joints was compared to the proteoglycan content in contralateral untreated joints.

Histology

Whole knee joints were dissected at different points of time after repeated intra-articular injection of 200 ng TGF-ß and processed as previously described (14). Semiserial frontal sections (6 μm) were mounted on gelatin-coated slides and stained with hematoxylin/eosin or safranin O/fast green for examination of cells and cartilage matrix, respectively. In addition to TGF-ß-induced changes, we also studied histology of naturally occurring murine OA in joints of 18-months-old mice, and of experimentally induced OA in collagenase-injected mice.

RNA isolation and RT-PCR

Total RNA was isolated from patellar cartilage and synovial tissue by TRIzol extraction. RNA was directly extracted from cartilage but synovial tissue was first homogenized and then put in TRIzol reagent (Life Technologies). Cartilage and synovium of 5 mice were pooled. Before reverse transcription the isolated RNA was treated with DNAse I (Life Technologies). The reverse transcription reaction was performed with Moloney murine leukemia virus (M-MLV) reverse transcriptase (Life Technologies) using an oligo$(Dt)_{15}$ primer (Eurogentec, Liege, Belgium). Amplification of DNA was accomplished by using Taq DNA polymerase (Life Technologies) up to a cycle number of 40. To determine the relative mRNA levels, 5 μl samples were taken at increasing cycle numbers. The PCR products were electrophorised in 1.6% agarose gels containing ethidium bromide. The cycle number at which the product was first detected on the gel was taken as a measure

for the amount of specific mRNA present in the originally isolated RNA. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) mRNA levels were used as an internal control. This method was validated by van Meurs et al. (15). All RT-PCR reactions were performed in duplicate. The following primers were used in the amplification reactions.
To detect GAPDH, the primers 5'-AACTCCCTCAAGATTGTCAGCA-3' (upper) and 5'-TCCACCACCCTGTTGCTGTA-3' were used (product 553 bp). Murine TGF-ß1 primers were derived from Clontech (Palo Alto, CA, USA)(product 525 bp), whereas murine TGF-ß2 (product 489 bp) and -ß3 (product 380 bp) were used as described by Mulheron et al. (16).

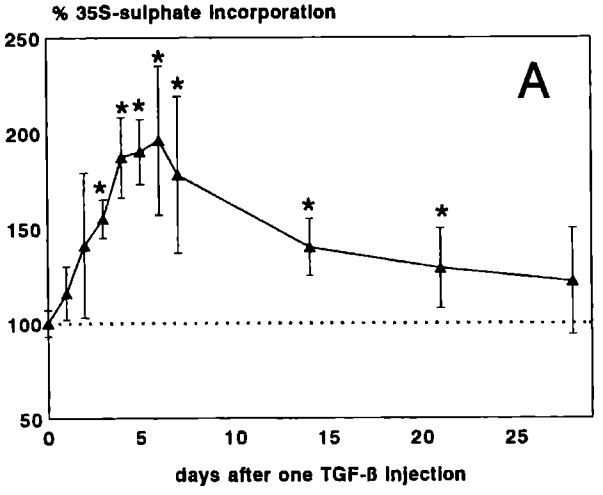
Statistical analysis

Differences between groups were tested using the Student's T-test. Differences were considered significant if $P < 0.05$.

RESULTS

Proteoglycan synthesis

Intra-articular injection of 200 ng TGF-ß stimulated articular cartilage proteoglycan synthesis after a lag time of about two days. Maximum synthesis (>200%) was reached after 4 days. Even three weeks after a single injection, proteoglycan synthesis was still significantly increased (Figure 1A). Three injections of 200 ng TGF-β (given at alternate days) did not further increase or prolong stimulation of proteoglycan synthesis as compared with a single injection of 200 ng TGF-β (Figure 1B).



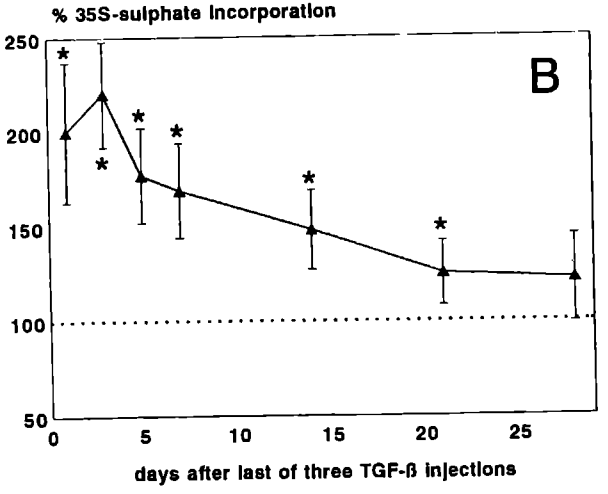


Figure 1. Long-lasting stimulation of proteoglycan synthesis in patellar cartilage after one injection with 200 ng TGF-β1 (A), or after three such injections at alternate days (B). Proteoglycan synthesis (^{35}S-sulfate incorporation) is expressed as percentage of that in the contralateral, vehicle-injected joints. The experiment was performed twice, with 6 patellae per experimental group at each point of time. Therefore the values represent the mean of 12 patellae.
** = $p < 0.05$*

Proteoglycan content

Densitometric analysis of safranin O staining intensity in histologic sections showed that the proteoglycan content in the superficial layer of patellar articular cartilage had significantly increased one week after injection of 200 ng of TGF-ß1 (Table 1). Stimulation of proteoglycan content was not further augmented when three injections of 200 ng TGF-β, in stead of a single injection, were given. The increase in proteoglycan content after three injections, lasted for three weeks whereafter the content slowly normalized.

The increase of proteoglycan content was not only found in patellar articular cartilage but was also demonstrated in articular cartilage of the lateral tibia. In the tibia, an increase in proteoglycan content was still measured 8 weeks after the last of three TGF ß injections (Table 1).

Table 1. Increase of safranin O staining in superficial articular cartilage after TGF-β injections

		patella		lateral tibia	
group	day[$]	n[&]	increase (% of control)	n	increase(%of control)
3X vehicle	D7	8	-11 ± 9		
1X TGF-β	D7	8	19 ± 10**		
3X TGF-β	D7	12	21 ± 6**		
3X TGF-β	D21	6	11 ± 5*		
3X TGF-β	D28	12	15 ± 11	12	18 ± 8*
3X TGF-β	D56	8	9 ± 7	8	12 ± 6*

Safranin O staining of articular cartilage in histologic sections was measured using an automated image analyser. The increase in staining intensity is expressed as percentage of the contralateral, non-injected control knee-joint.

[&] number of joints measured: of each joint at least three sections were measured.

Statistical significance of the TGF-β- induced increase was tested using the Student's t-test.

** $p<0.05$, ** $p< 0.01$ [$] time after the last of three injections.*

Lesions in tibial cartilage

Simultaneously with a supranormal proteoglycan content in the superficial layer, focal lesions with decreased safranin O staining developed deeper in the cartilage of the lateral tibia (Figure 2B). These lesions were found in an area just above the tidemark. Sometimes, the surface of the articular cartilage showed some roughening. In sections of several knee joints, a crack was found along the tidemark (Figure 2C). Interestingly, in early stages of natural murine OA, similar lesions were found. In later stages of murine OA noncalcified cartilage was lost appearing to have been teared of from the tidemark (Figure 2D). The TGF-β-induced lesions were already present one week after the last of three TGF-β injections, but their size increased in time. Lesions were not found in early frontal sections of the knee, but emerged in later sections of the posterior part of the joint. In a small percentage of control joints some proteoglycan loss was found in the same area, but the extent of these spontaneous lesions was minimal. No lesions were ever found in patellar cartilage. To study whether additional administration of

TGF-β further augmented the formation of TGF-β-induced lesions, experiments were performed in which 6 or 8 TGF-β injections were given. However, the additional injections did not elevate the formation of TGF-β-induced lesions.

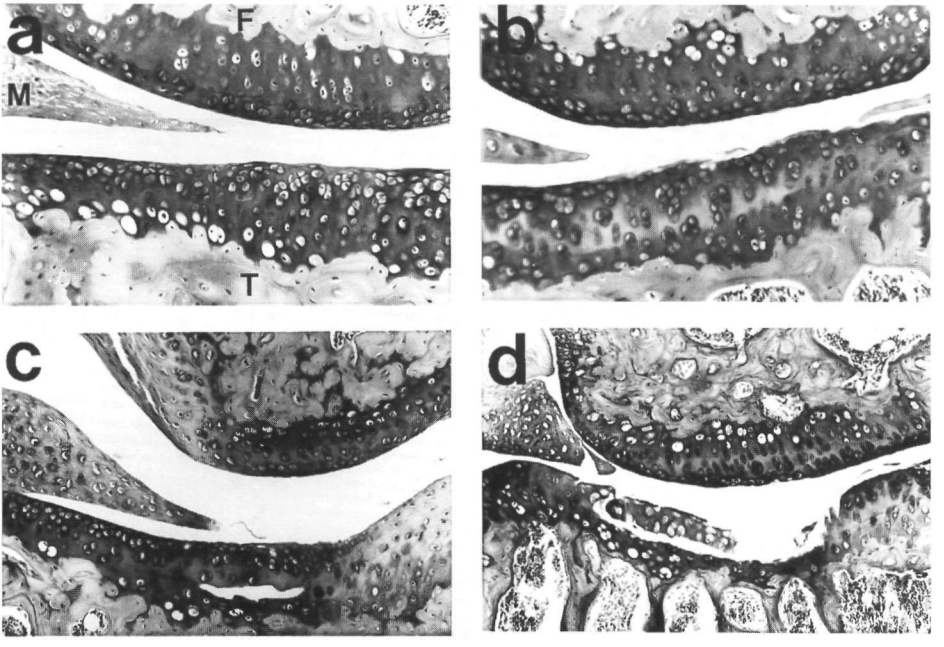


Figure 2. *TGF-β induced changes in lateral tibia. Safranin O/fast green stained frontal sections of murine knee joint.* ***A)*** *vehicle-injected control* ***B)*** *7 days after the last of three TGF-β-injections lesions with low proteoglycan content are present in the lateral tibia* ***C)*** *crack at the level of the tidemark in TGF-β-injected animal with lesion* ***D)*** *for comparison, spontaneously occurring crack at the level of the tidemark in a 18 months old mouse. F= femur, M = meniscus, T= tibia, Original magnification: 200 x*

Osteophytes

Triple injections of 200 ng TGF-ß induced activation of periosteum at the margins of articular cartilage. At these locations cartilage-like, proteoglycan-producing tissue developed (Figure 3B+C). These tissues, which are called chondrophytes, were not found after a single TGF-ß injection. One week after the last injection, the new tissues had reached their maximum size whereafter they calcified and developed into mature osteophytes, containing bone marrow spaces (Figure 3D). The periosteum remained responsive to TGF-ß, because two additional injections, given three weeks after the last of six injections, caused renewed development of cartilage-like tissue on the recently formed osteophytes (Figure 3E).

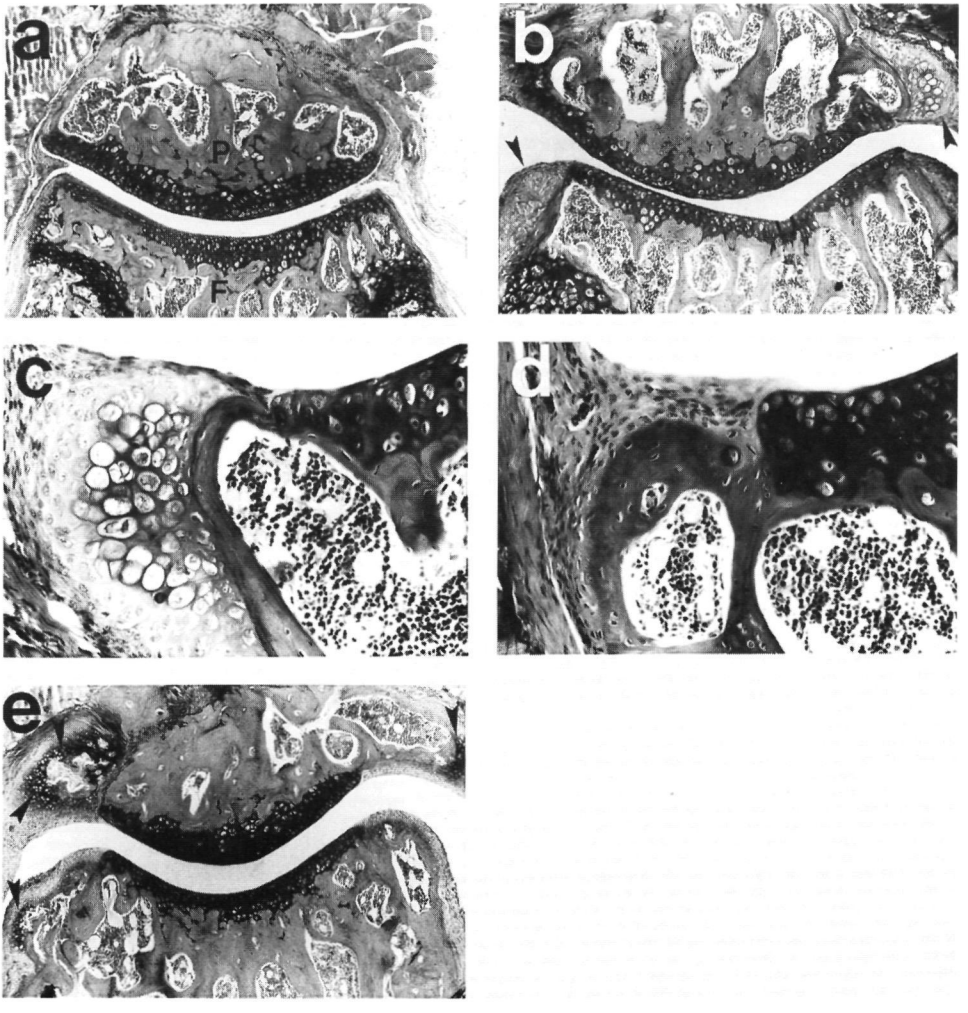


Figure 3. *Osteophyte induction by TGF-β.* ***A)*** *patella of vehicle-injected animal* ***B+C)*** *cartilage formation at the margins of articular cartilage of patella and femur, one week after third intra-articular injection of 200 ng TGF-β* ***D)*** *mature osteophytes, which do not bind safranin O any more and contain bone marrow spaces (one month after third injection) E) additional TGF-β injections restimulate periosteum of mature osteophytes (2 extra injections were given 3 weeks after the last of 6 injections; tissue was dissected one week after the last injection). Stained with safranin O/fast green. F = femur, P= patella, Original magnification: A,B and E: 100x, C and D: 400x*

Chondrogenesis in ligaments

Striking consequences of triple TGF-ß injections were synovial hyperplasia and thickening of ligaments and tendons inside and bordering the injected joint. If the joints were injected 6 or even 8 times the effects of TGF-β on cartilage proteoglycan content, focal proteoglycan depletion, and osteophyte development were not further extended compared to triple injections. However, in 9 out of 18 animals treated this way, safranin O staining indicated proteoglycan deposition in medial collateral ligaments. In addition, large rounded cells with lacunae and pericellular proteoglycans, presumably fibrochondrocytes, were found in these sites (Figure 4B). Interestingly, this phenomenon is also found in collateral ligaments of mice with collagenase-induced osteoarthritis (Figure 4C) and in collateral ligaments of mice with spontaneously occurring osteoarthritis.

TGF-β effects in old mice

To study whether TGF-β-induced chondrogenesis also occurs in old mice, intra-articular injections of TGF-β were given to 18-month-old mice. In these old mice, TGF-ß stimulated proteoglycan synthesis and induced the development of osteophytes. The effects were comparable to what was seen in young adults. This indicates that in old mice the chondrocytes and the cells of the periosteum still respond to TGF-ß. Moreover, TGF-ß injections appeared to induce development of cartilage-like tissue on already spontaneously formed bony structures (ossicles) in ligaments of 18-months-old animals with spontaneously occurring osteoarthritis (Figure 4D). These observations suggest that TGF-ß could be involved not only in induction, but also in outgrowth of such structures.

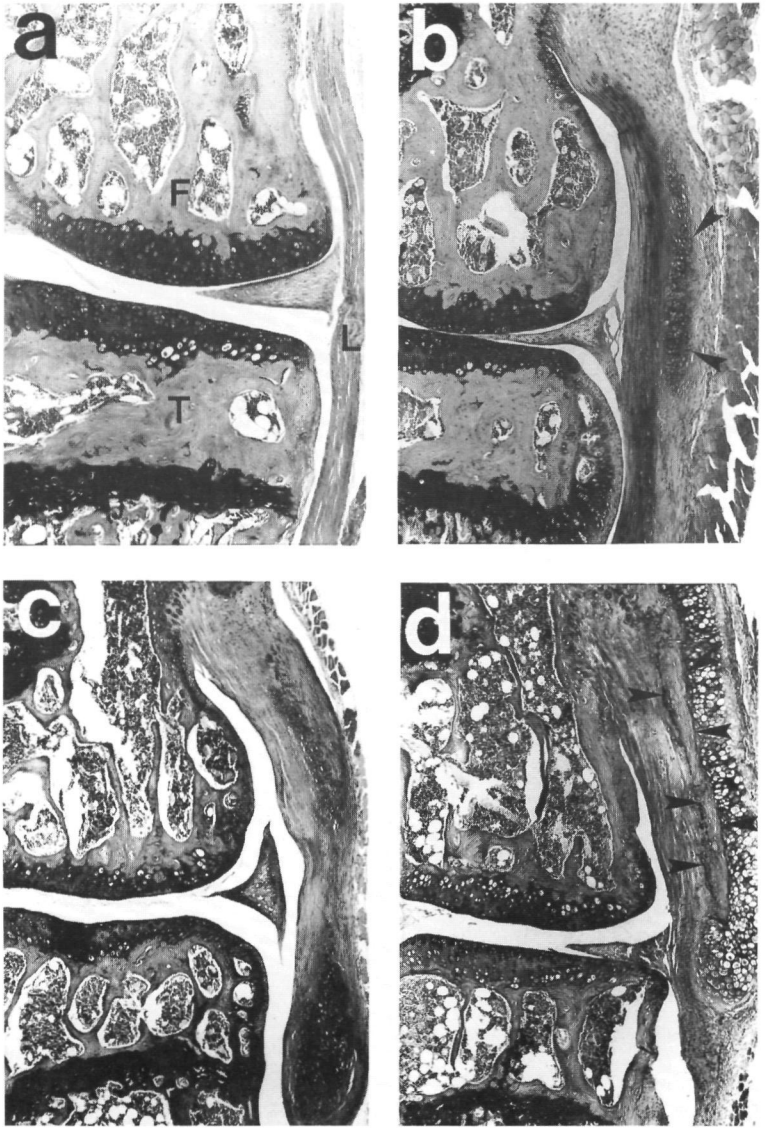


Figure 4. *Effects of TGF-β injections on medial collateral ligament.* ***A)*** *vehicle-injected joint* ***B)*** *cartilaginous tissue present in the ligament one month after 6 injections of TGF-β* ***C)*** *for comparison, cartilaginous tissue present in medial collateral ligament of mouse with collagenase-induced osteoarthritis. Stained with safranin O/fast green.* ***D)*** *TGF-β-induced activation of the surface cells of bony structure (ossicle marked by arrows) in medial collateral ligament in 18-months-old mouse with spontaneously occurring osteoarthritis; one week after third injection.*
F = femur, L = ligament, T = tibia. Original magnification: 100 x.

TGF-β autoinduction

Since TGF-ß has long-lasting effects on articular cartilage, we studied TGF-ß autoinduction in articular cartilage and neighbouring soft tissue. In patellar cartilage, mRNA levels of TGF-ß1, -ß2, and -ß3 were not elevated on D2 and D8 after three intra-articular injections with 200 ng TGF-ß1. In contrast, TGF-ß1, -ß2, and -ß3 mRNA levels were elevated in punches from neighbouring joint capsule on both points of time. In the TGF-ß1-injected knee joints TGF-ß1, -ß2, and -ß3 mRNA levels were elevated on both timepoints approximately 16-fold compared to control joints.

DISCUSSION

Previously, we have studied the short-term effects of intra-articular injections of TGF-β into murine knee joints. In these short-term studies, TGF-β stimulated articular cartilage proteoglycan synthesis and proteoglycan content (3,4). In addition, TGF-β induced development of cartilage-like tissues. Because both osteophyte formation and enhanced cartilage proteoglycan synthesis are also found in early experimental osteoarthritis (5,6) we hypothesized that TGF-β might be a causative factor in OA. Therefore we studied the long-term effects of intra-articular TGF-β injections on articular cartilage and other joint-tissues.

Intra-articular injections of TGF-ß induced, after a lag time of about 2 days, longlasting stimulation of articular cartilage proteoglycan synthesis. This suggests changes in chondrocyte phenotype, induction of second mediators, and/or TGF-β autoinduction (17,18). TGF-β mRNA induction was not detected in patellar cartilage, but strong and longlasting upregulation of mRNA of all three TGF-β isotypes was observed in adjacent joint capsule specimens after intra-articular injections of TGF-β1. This observation suggests that TGF-β induction in neighbouring soft connective tissue could be involved in the prolonged effect of TGF-β injections on chondrocyte metabolism.

In addition to enhanced proteoglycan synthesis, the proteoglycan content in the superficial layer of articular cartilage was also significantly increased after TGF-β injections. However, focal proteoglycan loss occurred in an area just above the tidemark, especially in the posterior part of the lateral tibia. In control knees similar lesions were sometimes found at the same location but those were much smaller. Cracks at the level of the tidemark, which are found in some TGF-ß-

injected joints, indicate that these regions are highly vulnerable to mechanical forces. Proteoglycan loss in the deeper part of articular cartilage after intra-articular injections with TGF-β seems to be a general feature since it has also been reported to occur in rats (19). Interestingly, we found similar lesions in early stages of collagenase-induced osteoarthritis and in old mice. In late stages of both natural and experimental osteoarthritis, noncalcified cartilage was either present or had disappeared completely, suggesting that tearing off at the site of the lesions is a normal feature of murine OA. In the literature, similar lesions in early stages of experimental osteoarthritis in mice, leading to fissuring at later time points, have been described (10,20,21). Moreover, spontaneous development of clefts at the level of the lateral tibial tidemark in C57 black mice which are genetically predisposed to develop osteoarthritis is also reported (22). Since elevation of proteoglycan synthesis and the formation of clefts in articular cartilage are induced by TGF-β and these changes are also features of both natural and experimental osteoarthritis, a role for TGF-β in the induction of OA is suggested.

As describe before (3, 23,24), TGF-ß injections in murine knee joints not only affected articular cartilage metabolism but also stimulated the periosteum, from which new cartilage developed. For development of these new structures multiple injections are needed since a single injection of TGF-β stimulated articular cartilage proteoglycan synthesis without inducing the development of these structures. This indicates that chondrocytes are more vulnerable to TGF-ß than periosteal cells are. The early cartilage-like structures develop into osteophytes and fuse with the original bone later on. The osteophytes were found principally along the margins of articular cartilage and at insertion sites of ligaments. Since osteophytes in spontaneously occurring and experimental osteoarthritis (3,10) are located on similar locations as osteophytes induced by TGF-β we hypothesize that TGF-ß is involved in osteophyte-formation during murine osteoarthritis.

Furthermore, thickening and the development of proteoglycan producing tissue was found in ligaments after 6 and 8 TGF-β injections which is in line with studies describing TGF-ß-induced proliferation and matrix synthesis by ligament fibroblasts (25,26). This proteoglycan-producing tissue could be the experimentally induced precursor of ossifications of ligaments found in spontaneously occurring osteoarthritis and is in line with the suggested role for TGF-ß in ossification of ligaments in humans (27).

We conclude that a single TGF-ß injection induces prolonged upregulation of articular cartilage proteoglycan synthesis and content, and could therefore be of therapeutic value. However, after multiple injections OA-like changes occur in cartilage and surrounding tissues. These latter observations suggest a pathogenic role for excessive local TGF-β production in osteoarthritis.

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CHAPTER 8

EXPRESSION OF RECOMBINANT HUMAN SOLUBLE TYPE II TRANSFORMING GROWTH FACTOR-β RECEPTOR IN PICHIA PASTORIS AND ESCHERICHIA COLI: TWO POWERFUL SYSTEMS TO EXPRESS A POTENT INHIBITOR OF TRANSFORMING GROWTH FACTOR -β

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ABSTRACT

Transforming growth factor β (TGF-β) is a potent regulator of cell metabolism, proliferation and differentiation. To study the role of endogenous TGF-β in processes such as tissue repair and inflammation, potent and specific inhibitors are required. Because the type II TGF-β receptor (TGFβRII) has a high affinity for TGF-β, the extracellular domain of TGFβRII (TGF-βsRII) was expressed in *Pichia pastoris* and *Escherichia coli*. Expression of the soluble TGFβsRII using *P. pastoris* resulted in a soluble, heterogeneously glycosylated protein which was secreted into the medium. Although expression of TGFβsRII in *E. coli* resulted in the formation of insoluble inclusion bodies, solubilization and refolding resulted in a biologically active protein. Because in both systems a C-terminal 6XHis coding sequence was inserted behind the coding sequence for the extracellular domain of TGFβRII the recombinant proteins could be purified by a powerful, single step procedure using a Ni-NTA agarose. The purified proteins appeared to be potent inhibitors of TGF-β1 and TGF-β3. In contrast, TGFβsRII was less effective in neutralization of TGF-β2.

In conclusion, biologically active TGFβsRII can be produced using *P. pastoris* and *E. coli* expression systems. The ease of these expression systems, the powerful single step purification and low costs makes it possible to produce TGFβsRII in large amounts to further elucidate the role of TGF-β1 and TGF-β3 in physiological processes like tissue repair and inflammation.

INTRODUCTION

Transforming growth factor β (TGF-β) is a potent regulator of metabolism, cell growth and differentiation (1). One of the physiological processes in which TGF-β plays an important role is tissue repair. It has been shown that TGF-β stimulates dermal wound healing (2,3), bone fracture healing (4) and cartilage repair (5-7). Although TGF-β has positive effects on tissue repair, overexpression of TGF-β might induce pathology. A role of TGF-β has been suggested in pathological processes such as intimal hyperplasia (8), glomerosclerosis (9-11), hepatic fibrosis (12), scarring during dermal wound healing (13,14) and articular cartilage lesions and osteophyte formation in joints (15-17). In addition to extracellular matrix production, TGF-β is also an important regulator of inflammation. Because TGF-β is a potent chemoattractant for monocytes, neutrophils and T-lymphocytes (18-20) it appears to be a proinflammatory factor. On the other hand, TGF-β has been shown to be immunosuppressive by inhibition of macrophage activity (21-23) and suppression of lymphocyte proliferation, differentiation and metabolism (24-28).

To study the role of endogenous TGF-β during disease, potent TGF-β inhibitors that can be produced in high levels are required. Because the type II TGF-β receptor (TGFβRII) has a high affinity for TGF-β (29,30) we expressed the soluble, extracellular domain of the type II TGF-β receptor (TGFβsRII). *Pichia pastoris* was used because this eukaryotic system has many of the advantages of higher eukaryotic expression systems such as protein processing, protein folding and posttranslational modifications but is more easy and cheaper to handle than other eukaryotic expression systems. *Escherichia coli* was used because of its high expression levels, ease of the system and low costs.

MATERIALS AND METHODS

Construction of a yeast expression vector

Vector H2-3FF containing the complete cDNA of human TGFβRII (30)(kindly provided by H. Lin) was used as a template for PCR amplification of the coding sequence of the extracellular domain of TGFβRII. PCR was performed using a high-fidelity DNA polymerase (Vent DNA polymerase, New England Biolabs, Beverly, MA, USA). The 5' primer used in the PCR was: 5'-AGACTCGAGAAAAGAGAGATCCCACCGCACGT TCAGAAG-3'. Using this primer a *Xho* I restriction site and a sequence coding for the cleavage signal of the *Saccharomyces cerevisiae* α-factor secretion signal peptide were introduced. The 3' primer was: 5'-GGAATTCAGTGATGGTGAATGGTGATGGTCAGG ATTGCTGGTGTTATATTCTTC-3'. By use of this primer a sequence coding for six consecutive histidine residues (6XHis tag) followed by a termination signal and an *Eco*RI restriction site were introduced. The PCR fragment was ligated in pCR-Script^{tm} SK(+) (Stratagene, la Jolla, CA, USA) whereafter the TGFβsRII-coding fragment was isolated after digestion with *Eco*RI and *Xho* I. This fragment was subsequently ligated into *Eco*RI/*Xho* I digested expression vector pPic-9 (Invitrogen, San Diego, CA, USA). The constructed vector (pPic-9/RII) contains an open reading frame coding for the *Saccharomyces cerevisiae* α-factor secretion signal and the entire sequence of the extracellular domain of TGFβRII. The sequence was confirmed by dideoxy chain termination sequencing.

Transformation of Pichia pastoris

Pichia pastoris strain GS115 was transformed using the spheroplast-method as described by Invitrogens protocols (Invitrogen Corporation, San Diego, CA, USA). Spheroplasts were incubated with Bgl II-linearized pPic-9/RII and transformants (His^+) were selected by their ability to grow on media lacking L-histidine. Because *Bgl* II digestion of pPic9/RII favors recombination at the alcohol oxidase gene AOX1, transformants were characterized for the presence of the AOX1 gene by the ability to grow on media with methanol as the only carbon source.

Expression of TGFβsRII by Pichia pastoris

Transformants were cultured at 30 °C in BMGY (1% yeast extract, 2% peptone, 1.34% yeast nitrogen base, 4 x 10^{-5} % biotin, 1% glycerol, 100 mM K-phosphate, pH6.0) until the turbidity at 600 nm reached >5. The cultures were centrifuged and the cell pellets were resuspended in BMMY (1% yeast extract, 2% peptone, 1.34% yeast nitrogen base, 4 x 10^{-5} % biotin, 0.5% methanol, 100 mM potassium phosphate, pH6.0) or BMM (1.34% yeast nitrogen base, 4 x 10^{-5} % biotin, 0.5% methanol, 100 mM potassium phosphate, pH6.0) until the turbidity at 600 nm reached 60-100. By culturing the cells in BMMY or BMM medium recombinant protein expression is induced since methanol activates the AOX1 promotor which controls recombinant protein expression. The cultures were grown for 2 days at 30 °C. To ensure adequate aeration baffled flasks were used and cultures were vigorously shaken (300 rpm). Media were assayed by an enzyme-linked immunosorbant assay (ELISA) using a polyclonal antibody against the first 28 N-terminal residues of human TGFβRII (Upstate Biotechnology Incorporated, New York, NY, USA).

Purification of TGFβsRII expressed by Pichia pastoris

The recombinant protein was purified by use of the 6XHis affinity tag that has a very high affinity to nickel-nitrilotriacetic acid (Ni-NTA; Qiagen Inc, Santa Clarita, CA, USA). Culture-medium (80 ml) was diluted 5 times with H_2O and pH was adjusted to 7.8. To reduce nonspecific binding to Ni-NTA imidazole was added to a final concentration of 5 mM. The medium was then applied to a 4 ml Ni-NTA agarose column with a flow rate of 0.5 ml/min. Subsequently, wash buffer (50 mM Na-phosphate, 300 mM NaCl, 10% glycerol, pH 6.0) was applied to the column and bound protein was eluted with 300 mM imidazole in wash buffer and collected in 1 ml fractions. The fractions were dialyzed against 50 mM Na-phosphate, 150 mM NaCl, pH 7.8 whereafter sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and isoelectric focusing (IEF) was performed. In addition, the neutralizing activity of the protein was determined using a bio-assay.

Deglycosylation of TGFβsRII

Purified TGFβsRII was dialyzed against deglycosylation buffer (50 mM EDTA, 1% β-mercaptoethanol, 1% n-octylglucoside, 0.2% SDS, 50 mM K-phosphate, pH 5.0) whereafter the sample was boiled to denaturate the protein. Endoglycosidase F/N-glycosidase F (Boehringer Mannheim, Mannheim, Germany), was added (2 U/mg protein) and the sample was incubated overnight at 37 °C. Samples were analyzed by SDS-PAGE.

Construction of bacterial expression vector

Vector H2-3FF containing the complete cDNA of human TGFβRII (30) (kindly provided by H. Lin) was used as an template for PCR. PCR was performed using a high-fidelity DNA polymerase (Vent DNA polymerase, New England Biolabs, Beverly, MA, USA) and two oligonucleotides: 5'-ATCGGATCCCACGTTCAGAAGTCGGTTAAT-3' and 5'-GAAGATCTGTCAGGATTGCTGGTGTTATATTCTTCTGA-3'. The PCR product contained a sequence corresponding to 97% of the extracellular domain of TGFβRII (aa3-aa114), an upstream *Bam*HI restriction-site and a downstream *Bgl* II restriction-site. The restriction-sites were used to introduce the fragment in the expression vector pQE-16 (Qiagen Inc, Santa Clarita, CA, USA). The resulting vector (pQE-16/RII) has an open reading frame coding for the extracellular domain of TGFβRII with 3 additional amino acids on the N-terminal site and a tag of six consecutive histidine residues (6XHis tag) on the C-terminus. The sequence of the open reading frame was confirmed by dideoxy chain termination sequencing.

Expression of TGFβsRII in Escherichia coli

E. coli strain M15[pREP4] (Qiagen Inc, Santa Clarita, CA, USA) was transformed with the vector pQE-16/RII and ampicillin resistant transformants were selected. For expression of the recombinant protein LB medium (10 g/l bacto-tryptone, 5 g/l yeast extract and 5 g/l NaCl) containing 100 μg/ml ampicillin and 25 μg/ml kanamycin was inoculated with a single colony. Bacteria were grown at 37 °C until the turbidity at 600 nm reaches 0.7-0.9. Expression of the recombinant protein was induced by addition of isopropyl-β-D-thiogalactopyranoside (IPTG) to a final concentration of 2 mM and the culture was grown for an additional 5 hours at 37 °C. Cells were harvested by centrifugation.

Purification and refolding of TGFβsRII expressed by E. coli

Purification of the recombinant protein was performed using Ni-NTA resin (Qiagen Inc, Santa Clarita, CA, USA) as this resin has a very high affinity to the C-terminal 6XHis tag of the protein. A 50 ml culture cell pellet was solubilized in 20 ml 8M urea (8M urea; 0.1M NaH_2PO_4, 0.01 M Tris, pH 8.0) whereafter the lysate was centrifugated (30 min, 12000xg, RT) and sonicated. Ni-NTA resin (4 ml) was added and after an incubation period of 1 hour at RT, the resin was collected by centrifugation (5 min, 800xg, RT) and washed for 3 times with wash-buffer (8M urea, 50 mM Tris/HCl, pH 8.0). Ni-NTA was resuspended in 10 ml wash-buffer and dialysed (o/n; 4 °C) against 1 liter refolding-buffer (0.1 M urea, 50 mM Tris/HCl pH 8.0) containing a redox couple of reduced glutathione (2 mM) and oxidized glutathione (0.2 mM or 0.02 mM) in the presence or absence of 0.5 M L-arginine. After dialysis Ni-NTA agarose was gathered and the recombinant protein was eluted by addition of 5 ml dialysisbuffer + 300 mM imidazole. After an incubation period of 5 minutes at RT the suspension was centrifugated (5 min, 800xg, RT) and the supernatant was subsequently dialyzed against 50 mM Na-phosphate, 150 mM NaCl, pH 7.8 (o/n, 4 °C) and analyzed by SDS-PAGE and IEF. In addition, the neutralizing activity was determined using a bio-assay.

SDS-PAGE

SDS-PAGE analysis was performed on 12 % polyacrylamide gels under denaturating conditions. Gels were stained with Coomassie brilliant blue R250. A 10 kDa protein ladder (Life Technologies, Gaithersburg, MD, USA) was used as a molecular mass marker.

Isoelectric focusing (IEF)

PhastGels IEF 3-9 (Pharmacia, Uppsala, Sweden) were used for IEF analysis. Proteins were visualized by silver staining whereafter isoelectric point (pI) of the recombinant proteins was determined using IEF standards ranging from pI 4.5 - 9.6 (Bio-Rad, Hercules, CA, USA).

NOB-1/CTLL TGF-β bioassay

To examine the neutralizing activity of TGF-ßsRII we used a modified bioassay for interleukin-1 (IL-1) which was originally described by Gearing et al. (31). In this modified assay TGF-ß inhibits the IL-1-dependent production of IL-2 by NOB-1 cells. Synthesis of IL-2 was assayed by proliferation CTLL cells, present in the same culture well as the NOB-1 cells. In microtiter plates NOB cells (100 μl of 2.5 x 10^5 cells/ml) and CTLL cells (50μl of 8 x 10^4 cells/ml) were plated out in RPMI medium supplemented with 5% fetal calf serum. In addition, IL-1β (25 μl of 500 pg/ml) and sample (25 μl containing TGF-β with or without TGFβsRII) were added to each well. After a culture period of 20 hours in a humidified 5% CO_2 atmosphere 1 μCi/ml ^{3}H-thymidine (du Pont de Nemours, Den Bosch, the Netherlands) was added. After a further 4 hours incubation, the cells were harvested by an automated cell harvester and the incorporated radioactivity was quantified by liquid scintillation counting. Commercially available TGFβsRII (expressed in mouse myeloma NSO cells; R&D Systems Inc, Minneapolis, MN, USA) was used as a positive control for TGF-β neutralization.

RESULTS

Expression of TGFβsRII by P. pastoris

To express TGFβsRII, an expression vector was constructed with an open reading frame coding for the extra-cellular domain of TGFβRII with an additional C-terminal α-factor secretion signal and a N-terminal 6XHis tag. The 6XHis tag was introduced to ease purification and the α-factor secretion signal is needed for efficient secretion of the recombinant protein. The secreted protein will not contain the α-factor secretion signal as this signal is cleaved during secretion.

After transformation of *P. pastoris* strain GS115 both Mut^s (containing no AOX1 gene) and Mut$^+$ (containing AOX1 gene) transformants were identified. Expression of TGFβsRII was assayed by analysis of media. Using an ELISA, positive clones were identified showing that TGFβsRII was expressed and secreted in the medium. Both Mut^s and Mut$^+$ clones were positive but the highest expression was found among Mut ^{s}clones. Maximal production of about 10 mg/l was reached after two days culture.

Purification of TGFβsRII produced by P. pastoris

Because a 6XHis tag was cloned behind the coding sequence for the extracellular domain of TGFβRII the recombinant protein could be purified from the culture medium by a single step procedure using a Ni-NTA agarose column. Purification appeared to be more effective from BMM medium as compared with BMMY medium suggesting that yeast extract and peptone negatively influence the binding of the recombinant protein to Ni-NTA. SDS-PAGE analysis of the purified protein did not show a distinct band but a band of 25 kDa with a smear up to 60 kDa was present (figure 1). To study whether this was the result of differential glycosylation of the three potential N-glycosylation sites, the protein was treated with glycosidases. As shown in figure 1, treatment with endoglycosidase F/N-glycosidase F resulted in a distinct band showing that TGFßsRII produced by *P. pastoris* is heterogeneously glycosylated. The molecular mass of the deglycosylated protein is about 22 kDa which is in line with the expected molecular mass of TGFβsRII. SDS-PAGE analysis of the deglycosylated protein showed that TGFβsRII was about 90% pure after a single purification step using Ni-NTA. The pI of purified, glycosylated TGFβsRII was determined by IEF analysis and appeared to be 7.0.

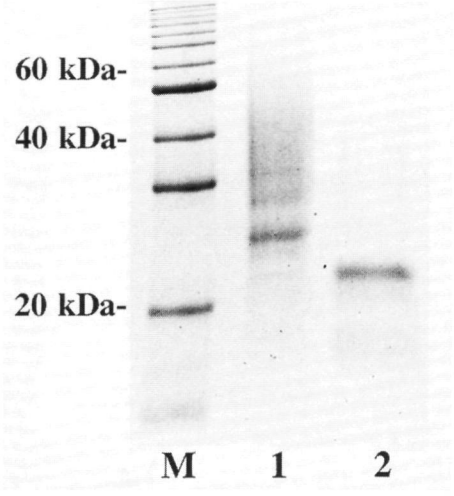

Figure 1 *SDS-PAGE analysis of TGFβsRII produced by P. pastoris before (lane 1) and after (lane 2) deglycosylation with endoglycosidase F/N-glycosidase F. SDS-PAGE analysis was performed on 12 % polyacrylamide gels under denaturating conditions. A 10 kDa protein ladder was used as a molecular mass marker.*

Expression of TGFβsRII by E. coli

Bacteria were transformed with a pQE-16 vector containing a sequence coding for the extracellular domain of TGFβRII with an additional 6XHis tag. Production of recombinant protein by transformants was induced by addition of IPTG and was assayed by SDS-PAGE. As shown in figure 2, addition of IPTG induced the expression of a recombinant protein with a molecular mass of 22 kDa. Maximal expression of about 15 mg/l was reached after 5 hours incubation in the presence of IPTG. Almost the entire fraction of TGFβsRII was insoluble, indicating that inclusion bodies were formed.

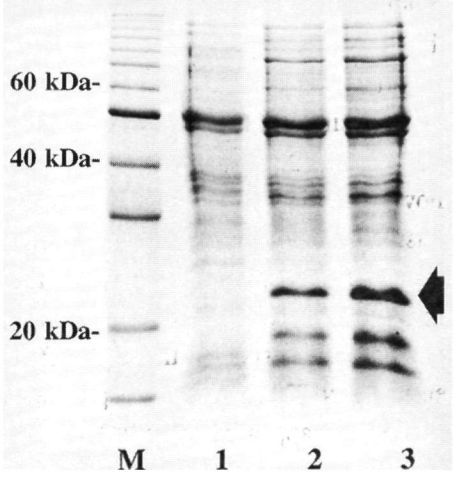

Figure 2 *Analysis of recombinant TGFβsRII production by E. coli after induction with IPTG. Samples of cultures were taken at t=0 (lane 1), t=3 hours (lane 2) and t= 5 hours (lane 3) after addition of IPTG. Samples were centrifuged and pellets were solubilized in 8M urea. SDS-PAGE was performed on 12 % polyacrylamide gels under denaturating conditions. A 10 kDa protein ladder was used as a molecular mass marker. The position of TGFβsRII is indicated with an arrow.*

Purification and renaturation of TGFβsRII produced by E. coli

After solubilization of the cell pellet in 8M urea, TGFβsRII was purified using Ni-NTA agarose in a batch procedure. As shown in figure 3 the purification procedure was very efficient and resulted in a >90% pure protein as determined by SDS-PAGE. To produce a functional TGF-β inhibitor the purified protein was renaturated. To prevent the formation of misfolded aggregates, refolding was performed while TGFβsRII was immobilized on Ni-NTA agarose. Several refolding procedures were used and the neutralizing activities of the proteins were tested in a TGF-ß bioassay. As can be seen in figure 4 the procedure in which refolding was performed in 2 mM reduced glutathione, 0.02 mM oxidized glutathione and 0.5M L-arginine was most effective in the production of a functional protein. Using IEF analysis the pI of purified and refolded TGFβsRII was shown to be 7.5.

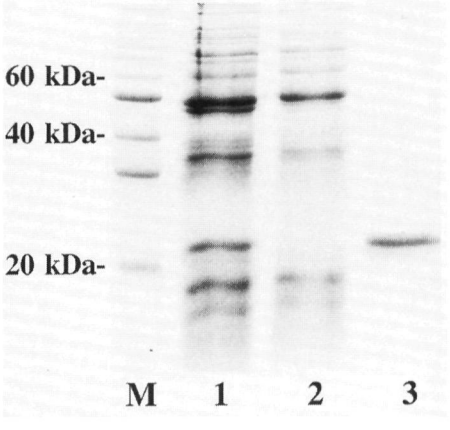

Figure 3 *Purification of TGFβsRII produced by E. coli. After a 5 hours culture period in the presence of IPTG, cells were solubilized using 8M urea and Ni-NTA agarose was added to the lysate. After an incubation period of 1 hour the cell lysate was removed and Ni-NTA was washed whereafter proteins were eluted using 300 mM imidazole. SDS-PAGE analysis was performed on a 12 % polyacrylamide gel under denaturating conditions. M: 10 kDa protein ladder; Lane 1: lysate before incubation with Ni-NTA agarose; Lane 2: lysate after incubation with Ni-NTA agarose; Lane 3: eluate from Ni-NTA agarose.*

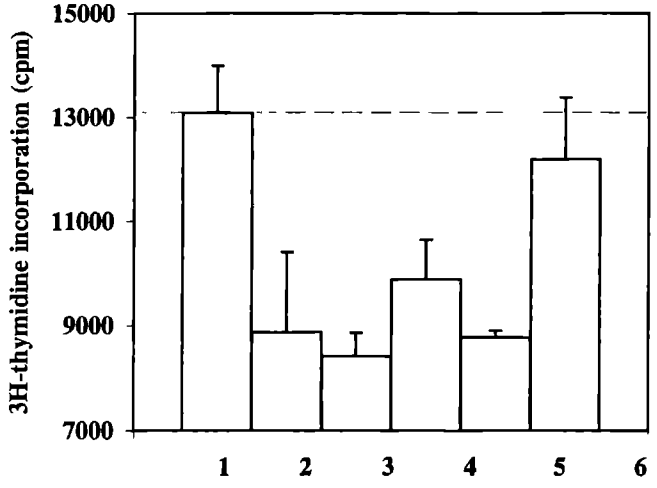

Figure 4 Effect of different refolding conditions on TGF-β neutralizing activity of E. coli TGFβsRII. TGFβsRII was refolded using reduced glutathione (2 mM) and oxidized glutathione (0.2 or 0.02 mM) in the absence or presence of 0.5 M L-arginine. After refolding, the neutralizing activity of TGFβsRII was determined using the NOB/CTLL bioassay in which TGF-β inhibits 3H-thymidine incorporation. Samples containing 3 ng/ml TGF-β1 and 100 µg/ml TGFβsRII were tested. 1: medium; 2: TGF-β; 3: TGF-β + TGFβsRII (2 mM reduced glutathione/0.2 mM oxidized glutathione) 4: TGF-β + TGFβsRII (2 mM reduced glutathione/0.02 oxidized glutathione) 5: TGF-β + TGFβsRII (2 mM reduced glutathione/0.2 mM oxidized glutathione + 0.5M L-arginine) 6: TGF-β + TGFβsRII (2 mM reduced glutathione/0.02 oxidized glutathione + 0.5 M L-arginine). 3H-thymidine incorporation in the absence of TGF-β is indicated by the dotted line.

Characterization of neutralizing activity of TGFβsRII

The neutralizing activities of TGFβsRII produced by *E. coli* and TGFβsRII produced by *P. pastoris* were compared using the NOB/CTLL bioassay. As shown in figure 5 both recombinant proteins were able to inhibit TGF-β1. However, TGFβsRII produced by *E. coli* appeared to be a more potent inhibitor than TGFβsRII produced by *P. pastoris*. Dose-response studies demonstrated that a 400-800 fold molar excess of TGFβsRII (*E. coli*) was required for complete neutralization of TGF-β1. A similar amount of commercially available TGFβsRII (expressed in NSO cells) was required for complete neutralization of TGF-β1 showing that the neutralizing activity of prokaryotic, refolded TGFβsRII (*E. coli*) and commercially available eukaryotic TGFβsRII were comparable (data not shown).

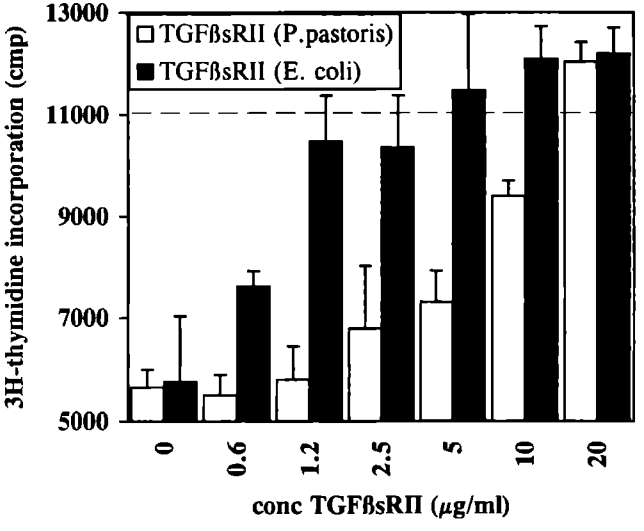

Figure 5 Comparison of neutralizing activities of TGFβsRII produced by E. coli and TGFβsRII produced by P. pastoris. Samples containing 3 ng/ml TGF-β1 and 0-20 μg/ml TGFβsRII were analyzed using the NOB/CTLL TGF-β bioassay. 3H-thymidine incorporation in the absence of TGF-β is indicated by the dotted line. Shown is a representative experiment of three.

In addition, the ability of TGFβsRII to inhibit biological activity of other mammalian TGF-β isoforms was studied. As shown in figure 6 TGFßsRII is not only a potent inhibitor for TGF-ß1 but is also a potent inhibitor of TGF-ß3. In contrast, TGFßsRII was less effective in neutralization of TGF-β2.

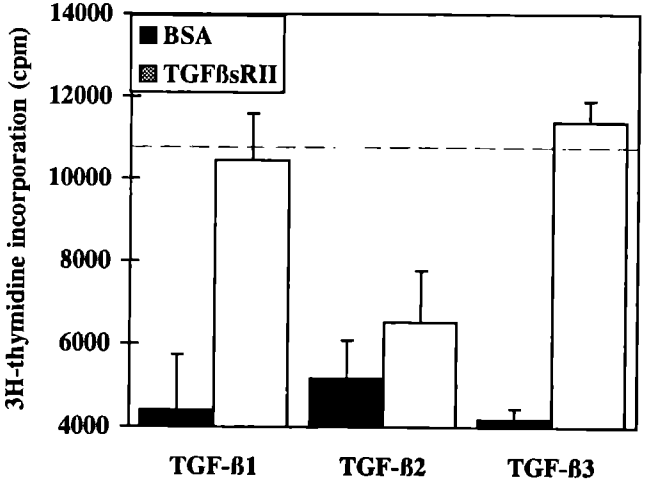

Figure 6 Determination of neutralizing activity of TGFβsRII for TGF-β1, TGF-β2 and TGF-β3. Samples containing 1.5 ng/ml TGF-β1, TGF-β2 or TGF-β3 in the presence of 30 μg/ml BSA or 30 μg/ml TGFβsRII were tested using the NOB/CTLL bioassay. 3H-thymidine incorporation in the absence of TGF-β is indicated by the dotted line. Shown is a representative experiment of three.

DISCUSSION

TGF-β seems to play an important regulatory role in inflammation and tissue repair. To study the role of endogenous TGF-β in pathological processes potent and specific TGF-β inhibitors are required. Because the soluble, extracellular domain of the type II TGF-β receptor (TGFβsRII) has been shown to neutralize TGF-β (32) we studied whether active TGFβsRII can be produced by *E. coli* and *P. pastoris*.

The yeast *P. pastoris* expressed and secreted heterogeneously glycosylated TGFβsRII. Comparable heterogeneous glycosylation was previously shown on TGFβsRII produced by NSO or COS cells (32,33). These results indicate that TGFβsRII produced by *P. pastoris* was not hyperglycosylated which is in line with other reports showing that *P. pastoris*, in contrast to other yeasts like *Saccharomyces cerevisiae*, does not hyperglycosylate recombinant proteins (34,35).

Expression of TGFβsRII in *E. coli* resulted in high levels ($\sim$ 15 mg/l) of insoluble recombinant protein. Since TGFβsRII was denaturated during solubilization, a renaturation procedure was required to obtain a biologically active protein. Unless very low protein concentrations are used, formation of misfolded aggregates often occurs during the renaturation process (36). To overcome this problem we performed refolding while the recombinant protein was immobilized on the Ni-NTA agarose. Formation of disulfide bonds was stimulated using a widely applied redox couple of reduced and oxidized glutathione (36-38). The efficiency of refolding appeared to be dependent on the ratio of reduced and oxidized glutathione used. Although a ratio of 10:1 is generally used (39), refolding of TGFβsRII was most efficient using a ratio of 100:1. The efficiency of refolding was clearly improved in the presence of 0.5M L-arginine. This is in line with other reports showing more efficient refolding of proteins in the presence of 0.5M L-arginine (36,40). TGFβsRII contains 12 cysteines, which are conserved between species (30,41), suggesting that they are all involved in the *Expression of TGFβsRII by P. pastoris* formation of disulfide linkages. Regardless of the high number of cysteines, *in vitro* refolding of TGFβsRII resulted in a protein with a TGF-β-neutralizing activity that is comparable with the neutralizing activity of commercially available, eukaryotic TGFβsRII.

The ability of TGFβsRII produced by *E. coli* and *P. pastoris* to neutralize TGF-β1 was compared. Both proteins were able to neutralize TGF-β showing that the C-terminal 6XHis tag does not eliminate the neutralizing activity of TGFβsRII. To our surprise, TGFβsRII produced by *E. coli* appeared to be a more potent inhibitor of TGF-β1 than

TGFβsRII produced by *P. pastoris*. Since it can be hypothesized that *in vitro* refolding of TGFβsRII might be inefficient due to the high number of conserved cysteines, this observation was unexpected. In addition, this finding was surprising as it has been described that unglycosylated TGFβsRII is about 40 fold less potent in binding TGF-β3 than glycosylated TGFβRII (42). Until now the explanation for the different neutralizing activities of TGFβsRII produced by *E. coli* and TGFβsRII produced by *P. pastoris* remains obscure.

TGF-βsRII appeared to be a potent inhibitor for TGF-β1 and TGF-β3 but was less effective in the neutralization of TGF-β2. This is in line with earlier reports that describe the low affinity of TGFβRII for TGF-β2 (32,33,43-45). The lower affinity for TGF-β2 might be the result of the different structure of TGF-β2 as compared with TGF-β1 and TGF-β3 (46-48).

In summary, TGFβsRII can be efficiently expressed by *P. pastoris* and *E. coli*. The proteins, produced by both expression systems, appear to be potent inhibitors for TGF-β1 and TGF-β3. Because of the ease and low costs of both systems, the high expression levels and the powerful one-step purification, TGFβsRII can now be produced and purified in high amounts to further study the role of TGF-β in inflammation, tissue repair and disease *in vitro* and *in vivo*.

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CHAPTER 9

SUMMARY AND FINAL CONSIDERATIONS

SUMMARY AND FINAL CONSIDERATIONS

Progressive destruction of articular cartilage is a main feature of osteoarthritis (OA) and rheumatoid arthritis (RA). The destruction of cartilage is the result of a disbalance between matrix synthesis and matrix degradation. In OA and RA degradation of matrix is enhanced due to elevated activity of proteinases produced by inflammatory cells, synovial tissue and articular chondrocytes. Besides enhanced degradation of matrix molecules, suppression of matrix synthesis also contributes to the process of cartilage destruction in arthritis. In contrast to arthritis, synthesis of matrix molecules is elevated in early OA but this is not able to overcome matrix degradation. Anabolic factors which are able to increase PG synthesis and decrease PG degradation might stimulate cartilage repair in arthritic and osteoarthritic joints. In this respect transforming growth factor β (TGF-β) and bone morphogenetic proteins (BMPs) seem promising.

TGF-β is a growth factor which is able to stimulate articular cartilage PG synthesis (1-3). However, the effects of TGF-β on PG synthesis of articular chondrocytes are contradictory since both stimulating and inhibitory effects of TGF-β on chondrocyte PG synthesis have been described (2,4,5). Studies were performed to investigate whether these differential effects of TGF-β are related to differences in the expression of TGF-β receptors. In **Chapter 2** TGF-β receptor expression of freshly isolated and cultured bovine articular chondrocytes was analyzed since we have previously shown that TGF-β inhibits PG synthesis of freshly isolated articular chondrocytes while PG synthesis of cultured chondrocytes is stimulated by TGF-β (5). Both types of chondrocytes expressed type I, type II and type III TGF-β receptors. Interestingly, the type II TGF-β receptor of cultured chondrocytes was about 15 kilodaltons smaller than the type II TGF-β receptor of freshly isolated chondrocytes. Because the type II TGF-β receptor appears to be directly involved in signal transduction, these results suggest that the differential TGF-β responses between freshly isolated and cultured chondrocytes are related to the differences in the size of the type II TGF-β receptor. Until now the mechanism of this action is unknown. Initially it was assumed that the type I and type II TGF-β receptors mediate distinct signal pathways independently. The type II TGF-β receptor seemed responsible for inhibition of chondrocyte metabolism while activation of the type I TGF-β receptor appeared to stimulate chondrocyte metabolism (6,7). We postulated that the small sized type II TGF-β receptor is a nonfunctional receptor leading to stimulating effects of TGF-β by activation of only type I TGF-β receptors. However, nowadays there is accumulating evidence that type I and type II TGF-β receptors signal through a heteromeric receptor

complex and that a functional type II TGF-β receptor is required for phosphorylation of the type I TGF-β receptor (8-10). It is not yet clear whether the type I and type II receptors in this complex mediate different signals as suggested by Chen et al (11) or that this complex induces only one signal pathway as is suggested by others (12-14). These new insights make it unlikely that the small sized type II TGF-β receptor is a nonfuctional receptor, as we initially postulated. Recently, 2 different isoforms of the type II TGF-β receptor have been described (15-18). It is unclear whether these isoforms are encoded by different genes or that they are generated by alternative splicing of a single gene. Because the difference in size between the "normal" type II TGF-β receptor and the small sized type II TGF-β receptor appears not to be the result of differences in glycosylation (non-published data) we assume that the small sized receptor is a new isoform. Hypothetically, a heteromeric receptor complex in which this isoform is included might phosphorylate different substrates leading to different cellular responses.

The discovery of a new isoform of the type II TGF-β receptor (TGFβRII$_2$)(15-17) prompted us to study the expression of this isoform in articular cartilage. This isoform contains an insertion of 25 amino acids in the extracellular domain. Although the function of TGFβRII$_2$ is unknown an important role of this isoform is suggested since this isoform is highly conserved between species (17). Therefore it was hypothesized that this isoform mediates TGF-β responses distinct from the 'normal' type II TGF-β receptor (TGFβRII$_1$) and that differential effects of TGF-β on chondrocytes might be the result of differences between the relative expression of these isoforms. As described in **Chapter 3** both TGFβRII$_1$ mRNA and TGFβRII$_2$ mRNA are expressed in human and murine articular cartilage. However, TGFβRII$_2$ mRNA was not detected in bovine articular cartilage. This implicates that it is unlikely that TGFβRII$_2$ plays an essential role in homeostasis of normal bovine articular cartilage. Because TGF-β responses appear to be age related (19-22) and differential TGF-β responses have been described between normal cartilage and cartilage undergoing repair (23-25), the relative expression of TGFβRII$_1$ and TGFβRII$_2$ was studied during cartilage repair and aging. No differences in the relative mRNA expression of the two isoforms could be demonstrated in murine articular cartilage during aging or during the repair phase after mild PG depletion suggesting that it is unlikely that differential TGF-β responses are the result of differences in the relative expression of the two isoforms.

Both BMP-2 and TGF-β have been shown to stimulate articular cartilage PG synthesis *in vitro* (1-3,26,27). To study the effects of these factors *in vivo*, they were directly injected into normal murine knee joints (**Chapter 4**). Both factors stimulated PG synthesis but intra-articular injection of BMP-2 induced a much earlier and more impressive stimulation of articular cartilage PG synthesis than intra-articular injection of TGF-β. However, one month after one injection of TGF-β PG synthesis was still significantly increased, while stimulation by BMP-2 only lasted for a few days. Stimulation of PG synthesis by TGF-β resulted in a prolonged enhancement of PG content while BMP-2, even after repeated administration, did not result in a long-lasting enhancement of PG content. To get insight in the effect of TGF-β and BMP-2 on mRNA expression of different PG types, RT-PCR was performed. TGF-β induced a very short stimulation of aggrecan mRNA expression while no effects on biglycan mRNA and decorin mRNA could be detected. BMP-2 stimulated mainly the expression of aggrecan mRNA but also the expression of biglycan mRNA and decorin mRNA. Remarkably, mRNA for collagen type X was unchanged after TGF-β exposure, but highly upregulated after BMP-2 injections. The latter observation suggests that BMP-2 triggers chondrocytes to differentiate into hypertrophic chondrocytes. After prolonged administration, both BMP-2 and TGF-β induced the formation of new chondroid tissues. These tissues were called chondrophytes. Interestingly, chondrophytes induced by BMP-2 seem to be derived from the growth plates while TGF-β-induced chondrophytes originate from the periosteum at sites remote from the growth plates.

It is assumed that IL-1 is a key mediator in the process of cartilage destruction. IL-1 seems to be responsible for suppression of PG synthesis during arthritis (28-30) and IL-1 is able to induce PG depletion after intra-articular injections into normal joints (31,32). The abilities of TGF-β and BMP-2 to counteract IL-1 effects on articular cartilage PG synthesis and content were compared (**Chapter 5**). Normal murine knee joints were injected with TGF-β or BMP-2 in combination with IL-1 and the effects on PG synthesis and content were analysed. TGF-β did not prevent initial IL-1-induced PG depletion but counteracted the effects of IL-1 on PG synthesis and clearly stimulated replenishment of proteoglycans in depleted matrix. Although BMP-2 is a very potent stimulator of articular cartilage PG synthesis it was, in contrast to TGF-β, not able to counteract the deleterious effects of IL-1 on articular cartilage PG synthesis and content. Since no significant effects of BMP-2 on chondrocyte PG synthesis could be demonstrated when chondrocyte metabolism is affected by IL-1 it is assumed that IL-1 induces BMP-2 unresponsiveness. Interestingly, BMP-2-induced formation of chondrophytes was not inhibited by IL-1.

TGF-β and BMP-2 appeared to be potent stimulators of articular cartilage PG synthesis *in vivo*. Therefore, the ability of these factors to stimulate articular cartilage repair in established arthritis was studied (**Chapter 6**). An unilateral arthritis was induced in mice by intra-articular injection of zymosan (heat killed *S. cerevisiae)*. Four days after the induction of arthritis three intra-articular injections with 200 ng TGF-β or BMP-2 were given at alternate days. Although it has been described that high levels of active TGF-β are present in human arthritic joints (33-36), local administration of TGF-β1 or TGF-β2 in murine arthritic joints clearly stimulated articular cartilage PG synthesis and stimulated replenishment of proteoglycans in depleted cartilage. Inflammation was not affected by the TGF-β injections. Unfortunately, TGF-β not only stimulated articular cartilage repair but also stimulated the formation of chondrophytes. The latter might limit therapeutic application of local TGF-β administration. In contrast to TGF-β, local administration of BMP-2 did not affect PG synthesis and no effects on PG content were detectable. The disability of BMP-2 to stimulate cartilage repair in arthritic joints might be attributed to the presence of IL-1 in these joints.

To elucidate the significance of possible side-effects, long-term results of local administration of TGF-β into normal murine knee joints were studied. As described in **Chapter 7**, the chondrophytes, which are induced after 3 injections of 200 ng TGF-β, do not disappear but calcify and develop into mature osteophytes. Moreover, histologic analysis of the articular cartilage in TGF-β-injected joints demonstrated areas with a decreased PG content in the cartilage of the lateral tibia. Because elevated PG synthesis, induction of osteophytes and PG depletion in cartilage of the lateral tibia are changes similar to changes observed in experimental and spontaneously occuring OA, these data suggest a role for TGF-β in the induction of OA. To study whether prolonged TGF-β administration induces real OA pathology, like fissures and irregularities at the cartilage surface, 8 injections of 200 ng TGF-β were given. This treatment did not extend PG depletion neither induced fissures or irregularities of the cartilage surface but induced chondrogenesis in ligaments. The latter is also a feature observed in spontaneously occurring OA. These data indicate that care must be taken with local application of TGF-β and that TGF-β might have a pathogenic role in the development of some aspects of OA.

To further investigate the role of endogenous TGF-β in the formation of osteophytes and the induction of OA, potent inhibitors for TGF-β are required. Because the type II TGF-β receptor (TGFβRII) has a high affinity for TGF-β (8,37,38), a soluble type II TGF-β receptor (TGFβsRII) was constructed and expressed by *Escherichia coli* and *Pichia pastoris* (**Chapter 8**). The ability of TGFβsRII to neutralize the biological activity of TGF-β was studied using bioassays. TGFβSRII appeared to be a potent inhibitor for TGF-β1 and TGF-β3 but was less efficient in neutralizing the biological activity of TGF-β2. Since high amounts of this inhibitor can be produced, *in vivo* studies can now be performed to elucidate the role of endogenous TGF-β in experimental arthritis and osteoarthritis.

In summary, this thesis demonstrates that local administration of TGF-β might be a promising treatment to stimulate articular cartilage repair in established arthritis. In contrast to TGF-β, BMP-2 is a very potent stimulator of articular cartilage PG synthesis but is not able to counteract IL-1 effects on PG synthesis and content and does not stimulate repair in established arthritis. Although TGF-β is a very potent stimulator of cartilage repair, therapeutic value might be limited due to the induction of osteophytes. However, future research might find ways to prevent the unwanted side effects by improving the protocol of TGF-β administration, pharmacological intervention or selective blocking of TGF-β responses in periosteum, synovium and ligaments. In contrast to the ability of TGF-β to stimulate cartilage repair, this thesis also showed a possible role of TGF-β in pathogenesis since OA-like changes are induced after prolonged administration of TGF-β in normal joints. Specific TGF-β inhibitors like TGFβsRII will be useful to further investigate the role of TGF-β in OA pathogenesis.

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HOOFDSTUK 10

NEDERLANDSE SAMENVATTING

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NEDERLANDSE SAMENVATTING

Articulair kraakbeen is een weefsel dat de uiteinden van de pijpbeenderen in een gewricht bekleedt. Het kraakbeen bestaat uit een veerkrachtige matrix dat de krachten die tijdens bewegen ontwikkeld worden goed kan opvangen waardoor het onderliggende bot tegen deze krachten beschermd wordt. Het kraakbeen zorgt er tevens voor dat gewrichtsoppervlakten met een minimale wrijving over elkaar kunnen glijden. De belangrijkste bestanddelen van de kraakbeenmatrix zijn proteoglycanen en collagenen. Deze eiwitten worden gemaakt door gespecialiseerde cellen die in de matrix liggen: de chondrocyten. Tijdens gewrichtsaandoeningen zoals reumatische artritis en osteoartritis vindt er degeneratie van het articulaire kraakbeen plaats. Dit resulteert in functieverlies van de gewrichten waardoor het vermogen om te bewegen beperkt wordt. Degeneratie van kraakbeen tijdens reumatische artritis is o.a. het gevolg van een verhoogde afbraak en een verlaagde aanmaak van proteoglycanen. In tegenstelling tot artritis is de aanmaak van proteoglycanen tijdens osteoarthrose hoger dan in normaal kraakbeen. Deze verhoogde aanmaak blijkt echter niet voldoende te zijn om de verhoogde afbraak van proteoglycanen te compenseren. Eiwitten die de afbraak van proteoglycanen kunnen remmen en de aanmaak van proteoglycanen kunnen stimuleren zouden een rol kunnen spelen bij het herstel van gedegenereerd kraakbeen. In dit proefschrift zijn twee groeifactoren nader onderzocht op hun vermogen het herstel van kraakbeen te stimuleren. Deze groeifactoren zijn: transforming growth factor-β (TGF-β) en bone morphogenetic protein-2 (BMP-2).

TGF-β blijkt chondrocyten te kunnen aanzetten om meer proteoglycanen produceren. Echter in sommige omstandigheden wordt de aanmaak van proteoglycanen door chondrocyten juist geremd onder invloed van TGF-ß. Om te achterhalen waarom TGF-β in bepaalde omstandigheden stimuleert en in andere omstandigheden juist remt, is onderzoek gedaan naar de aanwezigheid van TGF-β receptoren op chondrocyten. TGF-β receptoren zijn eiwitten die heel specifiek TGF-β kunnen binden. Chondrocyten hebben minstens 4 verschillende typen TGF-β receptoren (typen I,II,III en V). De type I en type II TGF-β receptoren kunnen door TGF-β geactiveerd worden waarna ze een signaal aan de cel doorgeven dat het gedrag van de cel doet veranderen. In **hoofdstuk 2** en **hoofdstuk 3** van dit proefschrift wordt beschreven dat de verschillende effecten van TGF-ß op chondrocyten niet in verband stonden met veranderingen in de aanmaak van 2 verschillende vormen van type II TGF-β receptoren (TGFβRII$_1$ en TGFβRII$_2$) door deze chondrocyten. Er leek echter wel een relatie te bestaan tussen het effect van TGF-β en de grootte van de type II TGF-β receptor op deze cellen.

Om de effecten van TGF-β en BMP-2 op de aanmaak van proteoglycanen door chondrocyten in een normaal gewricht te bestuderen, zijn deze factoren in het kniegewricht van muizen ingespoten. Zoals beschreven in **hoofdstuk 4** bleken zowel TGF-ß als BMP-2 de aanmaak van proteoglycanen in het kraakbeen flink te stimuleren. Opvallend is dat de stimulatie o.i.v. TGF-ß meer dan 3 weken aanhield terwijl BMP-2 slechts een stimulatie van enkele dagen gaf.

Interleukine-1 (IL-1) is een eiwit dat in verhoogde concentraties aanwezig is in zieke gewrichten en blijkt een belangrijke factor te zijn bij kraakbeendestructie. IL-1 kan namelijk de afbraak van proteoglycanen stimuleren en blijkt verantwoordelijk te zijn voor de geremde aanmaak van proteoglycanen tijdens artritis. Omdat IL-1 zo'n belangrijke rol speelt tijdens het proces van kraakbeendestructie is bestudeerd of TGF-β en BMP-2 de effecten van IL-1 op het kraakbeen kunnen tegenwerken. In **hoofdstuk 5** is beschreven dat het injecteren van IL-1 in een muizengewricht resulteerde in een geremde aanmaak van proteoglycanen en in een verminderd proteoglycaan-gehalte in het kraakbeen. Wanneer IL-1 en TGF-β tegelijkertijd werden geïnjecteerd bleek TGF-β niet in staat te zijn om te voorkomen dat het proteoglycaan-gehalte in het kraakbeen afneemt. Echter, TGF-β bleek wel heel duidelijk het herstelproces van het kraakbeen te stimuleren. Dit versnelde herstel was o.a. het gevolg van het feit dat TGF-β, zelfs in de aanwezigheid van IL-1, de aanmaak van proteoglycanen stimuleerde. Ondanks dat BMP-2 chondrocyten kon aanzetten om meer proteoglycanen te produceren (hoofdstuk 4), bleek BMP-2 in de aanwezigheid van IL-1 totaal geen effect te hebben op de aanmaak van proteoglycanen en het kraakbeenherstel. Kennelijk wordt de invloed van BMP-2 op chondrocyten geremd door de aanwezigheid van IL-1.

Omdat TGF-β en BMP-2 potente stimulatoren van de aanmaak van proteoglycanen door chondrocyten bleken te zijn, is onderzocht of deze factoren het herstel van kraakbeen in ontstoken gewrichten kunnen stimuleren. Hiervoor zijn TGF-β en BMP-2 ingespoten in ontstoken muizenkniegewrichten. Het kraakbeen in deze ontstoken gewrichten had een geremde aanmaak van proteoglycanen en een verlaagd proteoglycaan-gehalte. Zoals in **hoofdstuk 6** is beschreven, had het toedienen van BMP-2 geen duidelijke effecten op het kraakbeen. Dit zou mogelijk kunnen komen door de aanwezigheid van IL-1 in het gewricht. Het toedienen van TGF-β had wel duidelijk positieve effecten op het kraakbeen. Na injecties van TGF-β in artritische gewrichten bleek de aanmaak van proteoglycanen niet geremd maar zelfs hoger te zijn dan in normale gewrichten. Ook had het injecteren van TGF-β een positief effect op het proteoglycaan-gehalte van het kraakbeen. Dit gehalte was verlaagd in ontstoken gewrichten die niet met

TGF-β waren ingespoten maar was normaal in ontstoken gewrichten die ingespoten waren met TGF-β. De mate van ontsteking werd niet door TGF-β beïnvloed. TGF-β bleek dus on staat om, zelfs in de aanwezigheid van een ontsteking, kraakbeenherstel te stimuleren. Therapeutische toepassing van TGF-β wordt echter mogelijk beperkt doordat TGF-β ook de vorming van nieuw kraakbeenachtig weefsel (chondrofyten) in het gewricht stimuleerde.

In **hoofdstuk 7** zijn de lange-termijn effecten van het injecteren van TGF-β in normale gewrichten beschreven. De door TGF-β gevormde chondrofyten bleken niet te verdwijnen maar te veranderden in botstructuren (osteofyten). Ook werden andere pathologische veranderingen in het gewricht waargenomen. Zo bleek TGF-β de vorming van kraakbeenachtig weefsel in de pezen te veroorzaken en werd er in het articulaire kraakbeen plaatselijk een verlaagd proteoglycaan-gehalte aangetoond. Aangezien vergelijkbare veranderingen ook worden waargenomen in muizenkniegewrichten met beginnende osteoartritis, suggereren deze waarnemingen dat TGF-β betrokken zou kunnen zijn bij het onstaan van deze gewrichtsaandoening.

De rol van TGF-β bij het ontstaan van osteoartritis kan nader bestudeerd worden door de biologische activiteit van TGF-β te remmen. Dit kan door middel van eiwitten die aan TGF-β binden waardoor TGF-β niet meer in staat is om TGF-β receptoren te activeren. Omdat TGF-β sterk aan de type II TGF-β receptor bindt hebben we bacteriën (*E. coli*) en gisten (*P. pastoris*) zodanig verandert dat deze organismen het TGF-β bindende deel van de type II TGF-β receptor gaan produceren. De methoden die gebruikt zijn om de bacteriën en gisten te veranderen en om de geproduceerde eiwitten te isoleren zijn beschreven in **hoofdstuk 8**. De gezuiverde eiwitten blijken potente remmers te zijn voor TGF-β en kunnen gebruikt worden om de rol van TGF-β tijdens het ontstaan van osteoartrose nader te bestuderen.

Belangrijkste conclusies:

Ondanks het feit dat BMP-2 in normale gewrichten de chondrocyten stimuleert om meer proteoglycanen te maken, heeft BMP-2 geen effect op de aanmaak van proteoglycanen in de aanwezigheid van IL-1. Tevens bleek het injecteren van BMP-2 in ontstoken muizenkniegewrichten geen effect te hebben op de aanmaak van proteoglycanen en op het proteoglycaan-gehalte van het articulaire kraakbeen. In tegenstelling tot BMP-2 blijkt TGF-β de effecten van IL-1 wel heel goed te kunnen tegenwerken en heeft het toedienen van TGF-β in ontstoken gewrichten een duidelijk positief effect op het kraakbeen. TGF-β stimuleert namelijk, zelfs in de aanwezigheid van een ontsteking, de aanmaak van proteoglycanen en blijkt ook het proteoglycaan-gehalte van het articulaire kraakbeen weer te normaliseren. TGF-β lijkt dus een potentiele factor om het herstel van kraakbeen te bevorderen. Therapeutische toepassing van TGF-β wordt echter mogelijk beperkt doordat TGF-β ook de vorming van osteofyten stimuleert en dat het toedienen van TGF-β in een normaal muizenkniegewricht pathologische veranderingen tot stand kan brengen die vergelijkbaar zijn met veranderingen die waargenomen worden in een kniegewricht van een muis met beginnende osteoarthrose. Dit laatste suggereert dat TGF-β een rol zou kunnen spelen bij het ontstaan van osteoarthrose. Het beschreven onderzoek toont aan dat TGF-β het herstel van gedegenereerd kraakbeen kan stimuleren maar dat een teveel aan TGF-β ook een rol zou kunnen spelen bij het onstaan van kraakbeenschade in normale gewrichten.

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Harrie.

CURRICULUM VITEA

Harrie Glansbeek werd geboren op 23 januari 1963 te Breda. In 1980 behaalde hij zijn HAVO diploma aan scholengemeenschap Markenhagen te Breda. Hierna startte hij zijn studie aan de Nieuwe Leraren Opleiding (NLO) op het Mollerinstituut te Tilburg waar hij in 1985 zijn akte van bekwaamheid van de tweede graad tot het geven van voortgezet onderwijs in de vakken biologie en scheikunde behaalde. In datzelfde jaar begon hij met de studie Biologie aan de Katholieke Universiteit Nijmegen. Na stages op de afdelingen Hematologie (Academisch Ziekenhuis Nijmegen), Biochemie (Katholieke Universiteit Nijmegen) en Maag-, Darm-, en Leverziekten (Academisch Ziekenhuis Nijmegen) behaalde hij in 1989 het doctoraal examen. Vervolgens vervulde hij gedurende een periode van 18 maanden zijn vervangende dienstplicht op de afdeling Maag-, Darm-, en Leverziekten (Academisch Ziekenhuis Nijmegen). Vanaf 1991 was hij werkzaam op de afdeling Reumatologie van het Academisch Ziekenhuis Nijmegen waar hij begon met het onderzoek waarvan de resultaten in dit proefschrift beschreven staan. Sinds 1 september 1997 is hij werkzaam op de afdeling Virologie, vakgroep Infectieziekten en Immunologie van de faculteit Diergeneeskunde der Universiteit Utrecht.

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STELLINGEN

behorende bij het proefschrift

REGULATION OF ARTICULAR CHONDROCYTE PROTEOGLYCAN METABOLISM BY TRANSFORMING GROWTH FACTOR β AND BONE MORPHOGENETIC PROTEIN-2

I

IL-1α induceert BMP-2-ongevoeligheid bij articulaire chondrocyten (*dit proefschrift*).

II

TGF-β kan, zelfs in de aanwezigheid van een heftige gewrichtsontsteking, het herstelproces van articulair kraakbeen stimuleren (*dit proefschrift*).

III

Aangezien kraakbeen tijdens de vroege fase van osteoartritis gekenmerkt wordt door een verhoogde proteoglycaan-synthese en een verhoogd proteoglycaan-gehalte is het onwaarschijnlijk dat IL-1 een belangrijke rol speelt bij het ontstaan van deze ziekte.

IV

Het renatureren van sommige eiwitten verloopt efficiënter in de aanwezigheid van L-arginine (*dit proefschrift*).

V

Het voorkomen van een type II TGF-β receptor isovorm met een insertie van 25 aminozuren geeft aan dat het gen van de type II TGF-β receptor meer dan de 7 beschreven exonen moet bevatten.

VI

Indien ouders van spijbelende kinderen verplicht worden tot het volgen van opvoedkundige cursussen zal men spoedig ook met spijbelende ouders te maken krijgen.

VII

Het feit dat mobiele telefoons steeds vaker kinderlijke melodietjes produceren geeft aan dat deze apparaten vooral fungeren als speeltjes voor volwassenen.

VIII

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IX

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X

Vooruitgang is iets anders dan voortrazen op de ingeslagen weg (*Ode*).

XI

Pas nadat de eerste mens gekloond is, zal de maatschappelijke discussie omtrent klonen goed op gang komen.

XII

Bij verdere commercialisering van de wetenschap zullen straks ook wetenschappelijke presentaties onderbroken worden voor reclame.

Harrie Glansbeek
Nijmegen, 15 december 1997